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Obituary Notice

WILLIAM LEWIS DAVIES (1895–1941)

When William Lewis Davies died in India on 15 May, 1941, the world of dairy science lost one of its hardest workers in the prime of his powers. It is a loss we can ill afford for it was generally recognized that Davies was in a class of his own in dairy analytical chemistry. The writer has never known anyone with the facility in analysis that 'W. L.' possessed—for him to take samples of a number of cheeses in the morning and then come along with a complete nitrogen distribution before lunch was quite a commonplace. The other outstanding characteristic was an extremely wide knowledge of agricultural chemistry and he was frequently consulted by people in many kinds of industry and in many countries.

His career was a good example of success achieved without influence or family fortune. The son of a Carmarthenshire farmer, he was educated at Llandilo County School and on the outbreak of war in 1914 joined the Royal Horse Artillery and saw much active service in France. After demobilization he proceeded to University College, Aberystwyth, where he graduated B.Sc., with first class Honours in Chemistry. He later entered Gonville and Caius College, Cambridge, where he studied the constitution of proteins and received his Ph.D. degree in 1924. In 1925 he was appointed Advisory Agricultural Chemist at Reading University and proceeded to publish papers on the nitrogenous compounds of fish meal and the proteins of green forage plants. In 1927 he became Biochemist at The National Institute for Research in Dairying and soon established a reputation in dairy chemistry. His early work had been mainly on proteins but he now broadened his outlook and investigated many aspects of feeding stuffs. One of the most serious problems of the dairy industry was the deterioration of fat and Davies investigated the role of choline (from lecithin) and hydrogen peroxide in this fault. Coupled with fat breakdown was the problem of fishiness in butter to which he also made valuable contributions. The effect of metals and of sunlight on milk also claimed his attention. His method for the determination of chloride in milk is now standard and he also showed that traces of heavy metals could inactivate lipase in milk. In 1934 he was investigating the metabolism of betaine in relation to the use of beet pulp as a cattle food. During his stay at the Institute he was the joint author of a series of papers on the ripening of cheese.

In 1935 the University of Wales conferred the degree of D.Sc. on him in recognition of his contribution to dairy chemistry, and in 1936 was published the first edition of his *Chemistry of Milk* and this firmly established him as a leading authority on the subject.

In 1939 he was appointed Director of Dairy Research to the Government of India. Although stricken by illness shortly after his arrival in that country his drive and energy resulted in the organization of an active research centre (The Imperial Dairy Research Institute) which was officially opened on St David's Day, 1 March, 1941, only a few weeks before his death. It is a misfortune for India that Davies could not have been spared to impart his unique knowledge of dairying problems to the able workers that he had already gathered round him.

His death will be mourned by his many friends in the Biochemical Society, the Institute of Chemistry, the Society of Chemical Industry, the Royal Agricultural Society of England, the Agricultural Education Association and the British Dairy Farmers' Association. He is survived by his widow, son and daughter.

J. G. D.

63. Purification of Tetanus Toxoid

By **Peter Joseph Moloney** and **Joan Noreen Hennessy**
From the Connaught Laboratories, University of Toronto

(*Received 8 June 1942*)

There have been a number of reports [Parish & Oakley, 1940; Whittingham, 1940; Cooke, Hampton, Sherman & Stull, 1940], some unpublished, of anaphylactoid reactions in man following a second injection of tetanus toxoid. These reactions have been ascribed to proteoses or peptones present in the toxoid preparations; and it is highly probable that such constituents were responsible for the reactions observed. There is, however, the possibility that tetanus toxoid itself could serve as a sensitizing and shock-inducing agent and in view of this it was decided to explore the possibility of producing a suitable insoluble suspension of toxoid by precipitation, for example with alum or protamine [Moloney & Orr, 1938], in order to decrease its shock-producing properties. To make such an insoluble product it is desirable to have the toxoid in a relatively high state of purity, and to this end the method of purification which is described here was developed.

The method consists of first concentrating the toxoid solution and then precipitating the active material from the concentrated solution in the *p*H range 4·0–4·4. It might be objected, on the analogy of results obtained with diphtheria toxoid [Linggood, 1941], that subjecting tetanus toxoid to this degree of acidity would damage the toxoid protein. There is, however, no evidence of such damage in the experimental results which are presented.

Experimental

Details of the method

To 1500 ml. of crude toxoid contained in a 3-litre Erlenmeyer flask, 950 g. of ammonium sulphate are added. The flask is shaken until the ammonium sulphate is dissolved and then left overnight at room temperature. The solid which separates and floats to the surface of the liquid contains the greater part of the toxoid. The liquid is removed by careful decantation, and as it is poured off, the solid tends to adhere to the sides of the flask. We have never had occasion at this stage to resort to filtration. When the separation is completed, the precipitate remaining in the flask is dissolved in about 80 ml. of distilled water.

From this concentrated solution (50 ml. amount) the toxoid is precipitated by the addition of solid ammonium sulphate (30 g./50 ml. dialysed solution or corresponding amount calculated from Table 1). Acetate buffer *p*H 4·2 (125 ml.) is then stirred in over about 1 min. The mixture is at once centrifuged and the precipitate which contains the greater part of the toxoid is dissolved in about 30 ml. distilled water plus about 0·5 ml. 5% $NaHCO_3$ (to *p*H 7·0) and made up to 50 ml. The whole operation from the addition of the acetate buffer until the solution in bicarbonate should be completed within 10 min. The dissolved toxoid is centrifuged to free it from insoluble material and then dialysed against running water.

With the order of addition of ammonium sulphate and acetate buffer given above the best yields were obtained. There are however certain toxoid preparations, for example those containing Witte peptone, which on precipitation with ammonium sulphate yield sticky precipitates; these are not conveniently extracted with acetate buffer, and for such preparations it is preferable first to dissolve the ammonium sulphate in the buffer and then to add this solution to the concentrated toxoid.

In the above procedure it is required that ammonium sulphate be added to the concentrated solution of toxoid in the proportion of 30 g./50 ml. of ammonium sulphate-free toxoid solution. To this end, either the concentrated solution of toxoid can first be freed from ammonium sulphate by dialysis or the amount of ammonium sulphate required to be added can be determined from the specific gravity of the solution and the data given in Table 1. The figures given in the table are not absolute values, since, for example, there is a variation in the specific gravity of salt-free toxoid, but they are sufficiently accurate for the purpose in hand.

Table 1. *Specific gravity and ammonium sulphate required*

Specific gravity	g. $(NH_4)_2SO_4$ required per 50 ml. toxoid	Specific gravity	g. $(NH_4)_2SO_4$ required per 50 ml. toxoid
1·020	30·0	1·065	24·5
1·035	28·0	1·080	23·0
1·050	26·5		

N values of purified toxoids

The purities recorded in Table 2 may be compared with the Lf-nitrogen values for the crude toxoids which were purified, namely values ranging from 3·3 Lf/mg. N to 5·0 Lf/mg. N, and with the value for toxoid of 3300 Lf/mg. N derived from an analysis of

Table 2. *Yields and purities*

Toxoid	Original Lf/ml.	Purified Lf/ml.	Yield %	Purified Lf/mg. N
155	17	160	70	835
160	8	165	70	530
170	13	135	86	435
171	15	117	65	770
172	6	96	80	270
173	5	80	73	250
174	15	190	70	770
644	7	80	75	670
67	7	53	75	69

tetanus toxoid-antitoxin floccules [Moloney & Hennessy, 1942]. The method used in obtaining this latter figure was that described by Pappenheimer & Robinson [1937] for the analysis of diphtheria toxin-antitoxin floccules.

Table 3. *Reactivity of purified toxoids*

Toxoid	Peptone in crude toxoid	Result of purified toxoid intravenously	Toxoid	Peptone in crude toxoid	Result of purified toxoid intravenously
155	Martin's peptone	No anaphylaxis	173	Martin's peptone	No anaphylaxis
160	,,	,,	174	,,	,,
170	,,	,,	644	Neopeptone	,,
171	,,	,,	67	Witte peptone	Mild anaphylaxis
172	,,	,,			

Preparation no. 67 gave decidedly the poorest degree of purification. This result is in accord with the finding for the same toxoid, recorded in Table 3, namely that Witte peptone had not been effectively removed.

Presence of peptones in purified toxoids

Since it was highly probable, as noted above, that in some instances certain peptones were responsible for shock following a second injection of tetanus toxoid, it was of interest to test for the presence of these substances in the solutions of purified toxoid. This was

done by injecting the purified toxoids into guinea-pigs sensitized with the suitable peptones and noting whether or not anaphylaxis occurred.

The guinea-pigs which were used were sensitized as described by Moloney & Orr [1938] in testing diphtheria prophylactics. In the cases of Witte peptone and Neopeptone a 2% solution of the peptone was made and to this was added potassium alum to give a concentration of 1%. The *p*H of the solution was then adjusted with 5% Na_2CO_3 to approximately 6·0 and 5·0 ml. amounts of the suspension were injected subcutaneously into each of a number of guinea-pigs ranging in weight from 350 to 450 g. Six weeks after the injection of the alum-precipitated peptone the animals were ready for use. For sensitizing animals with Martin's peptone the same procedure was employed except that Martin's broth was used instead of a 2% peptone solution.

In carrying out a test the purified toxoid was first diluted to the same Lf value as the original toxoid and 1·0 ml. was injected into a femoral vein after exposure of the vein under local anaesthesia. The animal was observed for about 10 min. and if no reaction occurred its sensitivity was tested for by injecting 1·0 ml. of the original toxoid into the other femoral vein. In Table 3 'no anaphylaxis' means that following the intravenous injection of the purified toxoid there was no symptom of anaphylaxis, but that in the same animal following the intravenous injection of the original toxoid there was typical fatal anaphylaxis.

It should be noted that the presence of peptone in purified toxoids does not necessarily render these unsuitable for conversion into insoluble antigens, since the shock-producing properties of both peptone and toxoid are greatly lessened by precipitation with alum or other suitable compound.

Time of flocculation of purified toxoids

There are set forth in Table 4 the Kf values of original toxoids and of the corresponding purified toxoids. These latter were diluted to the original Lf values before flocculation.

Table 4. *Kf values of original and of purified toxoids*

Toxoid	Lf/ml. original and purified	Kf 46° original min.	Kf 46° purified min.
155	17	100	75
160	8	270	115
170	13	165	85
171	15	160	75
172	6	255	145
173	5	240	210
174	15	130	75
644	7	150	90
67	7	700	360

In every instance the times of flocculation of the purified toxoids are less than those of the original toxoids. On the basis of experience with diphtheria toxoid this result was unexpected since during purification the toxoids were exposed to an acidity represented by *p*H 4·2. Hence one might have looked for an alteration in the toxoid protein which would have been made manifest by an increase in the time of flocculation. One cannot of course conclude from the results which were obtained (Table 4) that the toxoids had suffered no damage; it is possible that the decrease in time of flocculation of the purified toxoids is due to the removal, during purification, of substances which tend to inhibit flocculation, a possibility which we have not explored.

Tetanus toxoid purified by the method described here has been used on a small scale to immunize animals and man, but as yet we have no adequate comparisons of the relative antigenic efficacies of purified and unpurified toxoids.

It is well known that flocculation with mixtures of tetanus toxin (or toxoid) and antitoxic sera may occur in more than one zone and that with, for example, a single serum

and a single toxoid, it is not possible by flocculation alone to decide which is the true toxoid-antitoxin zone. This difficulty can present itself with purified toxoid but in practice it has not arisen during a year in the routine testing of trial bleedings from horses undergoing immunization.

Summary

A simple method is described for the preparation of purified and concentrated solutions of tetanus toxoid. Purified toxoids made from broths containing Martin's peptone or neopeptone ranged in purity from 250 to 830 Lf/mg. N as compared with 3·3 Lf/mg. N to 5·0 Lf/mg. N for the original toxoids and 3300 Lf/mg. N as a probable value for pure toxoid.

The method of purification did not rid the toxoid of Witte peptone when this constituent was present in the crude toxoid. On the other hand crude toxoids containing Martin's peptone and neopeptone yielded products in which these peptones could not be detected.

The purified toxoids flocculated in less time than did the corresponding crude toxoids.

The authors wish to thank their colleague Mr M. D. Orr for making possible a comparison of flocculation and animal tests on trial bleedings from horses.

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64. Antibacterial Action and Chemical Constitution in Long-chain Aliphatic Bases

By **A. T. Fuller**, *From the National Institute for Medical Research, Hampstead, N.W. 3*

(*Received 25 June 1942*)

The success of sulphanilamide as a chemotherapeutic agent encourages the investigation of other classes of compound for antibacterial properties. The compounds described in this paper were the long-chain amines, amidines and guanidines which had been shown by King, Lourie & Yorke [1938] to possess remarkable trypanocidal powers. They were chosen because they had a proved biological action, and also formed a convenient series of related compounds in which the effects of changes in constitution on antibacterial action could be observed. These compounds were generously placed at my disposal by Dr Harold King, to whom my grateful thanks are due.

The drugs are listed below. Many of them have powerful antibacterial actions, and the effects of variations in the length of carbon chain, in the end groupings and in the bacteria studied are discussed.

Drugs. These comprised the hydrochlorides of:

1. Diamines, general formula $NH_2.(CH_2)_n.NH_2$, with $n=2$, 6, 14, 16 and 18.
2. Diamidines, general formula $NH_2.(NH:)C.(CH_2)_n.C(:NH)NH_2$, with $n=8$, 9, 10, 11, 12, 13, 14, 16 and stilbene diamidine.
3. Diguanidines, general formula $NH_2.(NH:)C.NH.CH_2)_n.NH.C(:NH).NH_2$, with $n=5$, 6, 8, 10, 12, 14, 16 and 18.
4. Di-*iso*thioureas, general formula $NH_2.(NH:)C.S.(CH_2)_n.S.C.(:NH)NH_2$, with $n=4$, 6, 8, 11, 12 and 16.
5. Di-quaternary ammonium compounds, general formula $\overset{+}{N}(CH_3)_3.(CH_2)_n.\overset{+}{N}(CH_3)_3$, with $n=8$ and 14.
6. Monoamines, general formula $H.(CH_2)_n.NH_2$, with $n=9$, 12, 14, 16 and 18.
7. Monoamidines, general formula $H.(CH_2)_n.C(:NH).NH_2$, with $n=15$ and 17.
8. Monoguanidines, general formula $H.(CH_2)_n.NH.C(:NH)NH_2$, with $n=9$, 14, 16 and 18.
9. Mono-quaternary ammonium compounds, general formula $H.(CH_2)_n.\overset{+}{N}(CH_3)_3$, with $n=9$ and 18.
10. Phenyl diguanide, phenyl guanidine, hexamethylene dipiperide, and octamethylene di-imino ether were inactive at the concentrations tested.

For brevity, in the text, 18-methylene diamine, e.g., is referred to as 18-diamine, or as $n=18$. It should be noted that since, by the above nomenclature, one carbon atom is included in the amidine group, the diamidines have $n+2$ and the monoamidines $n+1$ carbon atoms in an unbroken chain. The 12-diamidine, for example, is therefore compared with the 14-diamine, and the 15-amidine with the 16-amine.

Methods

The activity of the drugs is recorded (Tables 1–4) as the limiting dilution in thousands which prevents visible growth after 18 hr. incubation at 37° in Hartley digest broth, or (when testing the effect of serum) in a mixture of equal parts of inactivated human serum and Hartley broth. *V. cholerae* grew with difficulty in 50% serum broth, so 33% was used for this organism. The drug was added with aseptic precautions from stock dilutions of 1 in 500, 1 in 5000, 1 in 50,000 or 1 in 500,000, as required, and a control tube of medium was always included. The determinations were repeated on either side of the

limiting concentration, to check this up. Altogether more than 15,000 tests were done in this series. The implant was 5 μl. of a 1 in 200 dilution of overnight culture in 100 μl. of medium. This heavy inoculum was used to minimize variations due to differences in the implant.

Table 1. *Limiting inhibitory dilutions of drugs in thousands against cocci in broth and 50% serum broth*

	Str. viridans		*Str. pyogenes*		*Pneumococcus*		*Staph. aureus*	
Drug	Broth	Serum	Broth	Serum	Broth	Serum	Broth	Serum
6-Diamine	0·2	0·1	0·3	0·08	—	—	—	—
14- ,,	1500	500	200	500	80	300	20	30
16- ,,	4000	200	400	100	1500	40	100	20
18- ,,	7000	200	2000	100	2000	30	200	20
8-Diamidine	5	5	20	20	5	5	3	20
9- ,,	10	5	50	30	15	10	7	40
10- ,,	20	10	100	50	20	15	20	80
11- ,,	50	20	80	200	50	20	50	100
12- ,,	200	20	80	1000	50	20	100	400
13- ,,	500	100	100	1000	100	50	200	500
14- ,,	600	100	200	1000	200	50	200	800
16- ,,	1000	50	300	300	100	50	200	200
Stilbene-diamidine	100	20	150	150	100	20	30	100
5-Diguanidine	1	3	8	2	0·5	1	1·5	10
6- ,,	3	5	50	10	3	2	3·5	20
8- ,,	20	30	200	150	30	15	15	70
10- ,,	100	30	300	300	80	30	80	300
12- ,,	250	200	400	1500	150	50	700	500
14- ,,	5000	200	2000	1000	4000	200	2000	500
16- ,,	2000	200	1000	500	300	150	500	300
18- ,,	4000	100	2000	200	500	100	500	150
4-Di-*iso*thiourea	1000	10	500	50	50	15	100	5
6- ,,	2	5	10	50	10	10	3	7
8- ,,	1000	100	300	100	150	50	200	<10
11- ,,	300	100	200	100	400	100	20	10
12- ,,	200	50	300	150	30	40	10	5
16- ,,	800	200	1000	80	150	50	100	10
8-Di-quaternary base	0·1	<0·1	0·25	<0·1	<5	<5	<5	5
14- ,,	15	3	20	150	<5	<5	<5	40
9-Amine	150	70	20	100	40	20	5	20
12- ,,	1500	40	700	50	7000	10	150	10
14- ,,	3000	80	500	20	1000	10	150	10
16- ,,	1000	20	250	20	200	<20	75	<20
18- ,,	150	20	15	<20	200	20	15	<20
15-Amidine	150	100	500	20	200	15	400	<20
17- ,,	100	100	75	<20	100	<20	20	<20
9-Guanidine	300	30	100	100	50	20	20	50
14- ,,	500	50	500	75	200	20	100	30
16- ,,	800	30	500	50	200	<20	200	20
18- ,,	150	20	100	20	100	<20	150	<20
9-Quaternary base	10	20	10	100	5	10	2	10
18- ,,	200	20	150	15	150	20	100	20

Cultures

The main tests were carried out with the following 20 cultures:

Streptococcus viridans, 'B 37' (stock strain) from Dr S. D. Elliott.
Streptococcus pyogenes, group A, type 3, 'Richards' (mouse virulent).
Staphylococcus aureus, 'Paler' (recently isolated).
B. proteus, 'Hunt' (recently isolated).
B. pyocyaneus, 'Scudder' (recently isolated), all from Dr L. Colebrook.
Pneumococcus, type 2 (mouse virulent).
Pasteurella, 265 (mouse virulent), both from Dr van den Ende.

Cl. oedematiens, 'Cossard'.
Vibrion septique, 'V.S. 20'.
Cl. welchii, 'Rosher'.
Cl. tertium, 'WPRL', all stock strains from Dr H. J. Parish.
Cl. sporogenes, 'A. 37' (recently isolated), from Prof. A. A. Miles.
Bact. shigae, 'Newcastle' (stock strain).
Bact. flexneri, W (recently isolated).
Bact. sonnei (recently isolated).
Bact. typhosum, Felix type 2 (Vi++).
Bact. paratyphosum B, Felix type H 33 (Vi++).
Bact. typhi-murium (recently isolated).
Bact. enteritidis, 'Dublin' (recently isolated).
} All from the late Dr W. M. Scott.
Vibrio cholerae, 'Inabe S' (stock strain) from Mr P. Bruce White.

Some of the results were checked with dysentery cultures kindly supplied by Col. H. T. Findlay.

Table 2. *Limiting inhibitory dilutions of drugs in thousands against anaerobes in broth and 50% serum broth*

	Cl. oedematiens		*Vib. septique*		*Cl. sporogenes*		*Cl. tertium*		*Cl. welchii*	
Drug	Broth	Serum	Broth	Serum	Broth	Serum	Broth	Serum	Broth	Serum
6-Diamine	—	—	—	—	—	—	—	—	—	—
14- ,,	30	100	30	80	50	150	30	40	10	40
16- ,,	200	150	200	150	250	250	250	250	150	80
18- ,,	700	50	400	20	400	40	250	50	500	40
8-Diamidine	5	<5	<5	<5	<5	<5	<5	<5	<5	<5
9- ,,	10	<5	<5	<5	<5	<5	<5	<5	<5	<5
10- ,,	15	5	5	<5	<5	<5	<5	<5	<5	<5
11- ,,	50	7	5	<5	<5	<5	<5	<5	5	<5
12- ,,	50	15	20	4	20	5	5	<5	5	<5
13- ,,	50	30	40	5	25	7	25	5	10	5
14- ,,	100	50	150	10	100	20	60	7	10	10
16- ,,	200	40	150	20	250	20	100	10	50	20
Stilbene-diamidine	50	7	<20	5	<20	5	<20	<5	<20	<5
5-Diguanidine	—	—	—	—	—	—	—	—	—	—
6- ,,	—	—	—	—	—	—	—	—	—	—
8- ,,	5	<5	<5	<5	<5	<5	<5	<5	<5	5
10- ,,	10	10	20	<5	10	5	20	<5	7	20
12- ,,	30	20	40	10	30	20	20	15	30	30
14- ,,	700	150	500	50	600	100	500	15	300	100
16- ,,	400	150	200	10	500	20	300	20	300	50
18- ,,	500	50	300	20	600	40	400	15	300	50
4-Di-*iso*thiourea	75	5	20	5	75	4	40	<5	20	4
6- ,,	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
8- ,,	75	5	50	5	75	<5	100	<5	50	5
11- ,,	20	10	10	10	20	5	20	5	10	10
12- ,,	20	7	20	7	20	<5	10	4	5	5
16- ,,	50	10	20	5	150	5	40	7	10	5
8-Di-quaternary base	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
14- ,,	20	7	<5	<5	<5	<5	<5	<5	<5	<5
9-Amine	25	10	35	20	10	10	10	40	10	10
12- ,,	350	30	150	20	150	20	350	40	200	20
14- ,,	800	20	400	10	200	20	350	20	200	10
16- ,,	400	20	600	20	350	<20	350	20	150	<20
18- ,,	400	<20	150	<20	75	<20	75	20	75	<20
15-Amidine	400	<20	100	<20	200	<20	200	<20	150	<20
17- ,,	100	<20	75	<20	50	<20	100	<20	100	<20
9-Guanidine	75	20	75	5	75	5	35	<5	20	15
14- ,,	100	10	100	10	200	<10	100	10	100	10
16- ,,	50	20	200	<20	100	<20	100	<20	200	<20
18- ,,	150	<20	20	<20	200	<20	75	<20	150	<20
9-Quaternary base	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
18- ,,	75	10	150	5	100	5	150	5	100	5

Table 3. *Limiting inhibitory dilutions of drugs in thousands against colon-typhoid organisms in broth and 50% serum broth*

Drug	*B. typhosum*		*B. enteritidis*		*V. cholerae*		*B. paratyphosum* B		*B. typhi-murium*	
	Broth	Serum	Broth	Serum	Broth	Serum	Broth	Serum	Broth	Serum
6-Diamine	<0·1	0·1	—	—	—	—	—	—	—	—
14- ,,	20	50	10	20	10	40	10	30	10	20
16- ,,	100	30	50	30	50	30	50	20	50	20
18- ,,	100	20	15	4	100	20	35	7	15	5
8-Diamidine	0·8	7·5	<5	<5	<5	<5	<5	<5	<5	<5
9- ,,	1·2	15	<5	4	<5	<5	<5	<5	<5	<5
10- ,,	4	50	5	15	<5	7	<5	5	<5	5
11- ,,	7	35	7	20	<5	10	<5	8	<5	5
12- ,,	30	100	20	30	10	15	5	15	<5	10
13- ,,	50	120	20	50	20	30	10	20	5	20
14- ,,	75	100	30	20	20	15	30	15	10	10
16- ,,	100	30	30	10	20	15	30	7	20	5
Stilbene-diamidine	60	200	35	40	<20	20	<20	40	<20	40
5-Diguanidine	0·3	2	—	—	—	—	—	—	—	—
6- ,,	0·5	7	—	—	—	—	—	—	—	—
8- ,,	5	20	<5	5	<5	<5	<5	<5	<5	<5
10- ,,	40	200	5	20	5	10	7	10	<5	5
12- ,,	70	300	20	50	20	20	15	20	5	15
14- ,,	300	200	150	50	50	20	40	20	25	20
16- ,,	700	80	400	40	150	15	120	10	100	5
18- ,,	500	50	300	20	200	20	200	10	150	<10
4-Di-*iso*thiourea	20	15	20	<5	20	5	20	<5	20	5
6- ,,	0·8	15	<5	<5	<5	<5	<5	<5	<5	<5
8- ,,	200	10	100	<10	200	<10	100	<10	100	<10
11- ,,	30	40	25	15	10	15	10	15	15	10
12- ,,	10	20	10	10	5	7	5	15	10	10
16- ,,	50	35	25	20	20	20	25	20	30	20
8-Di-quaternary base	<0·1	<0·1	<5	<5	<5	<5	<5	<5	<5	<5
14- ,,	0·5	4	<5	4	<5	<5	<5	<5	<5	<5
9-Amine	7	7	7	10	5	10	5	10	5	7
12- ,,	100	10	50	<5	150	10	60	<5	80	5
14- ,,	120	<10	20	<10	300	10	35	<10	30	<10
16- ,,	<10	<20	<10	<20	20	<20	<10	<20	<10	<20
18- ,,	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
15-Amidine	200	<20	50	<20	200	<20	50	<20	40	<20
17- ,,	<20	<10	<20	<20	20	<20	<20	<20	<20	<20
9-Guanidine	75	20	50	15	20	7	10	10	20	7
14- ,,	200	10	100	<10	200	<10	40	<10	50	<10
16- ,,	<20	<10	<20	<10	50	<10	<20	<10	<20	<10
18- ,,	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
9-Quaternary base	2	5	<5	5	<5	5	<5	<5	<5	<5
8- ,,	5	<0·5	<5	<5	75	<5	<5	<5	<5	<5

Activity in broth

Sensitivity of bacteria. Bacteria differed greatly in their sensitivity to the drugs (Tables 1–4). For example 18-diamine inhibited *Str. viridans* at 1 in 7,000,000, *Staph. aureus* at 1 in 200,000, *B. enteritidis* at 1 in 15,000 and was inactive against *B. proteus*. In general, if the bacteria are arranged according to their sensitivities, the order in which they fall is roughly the same for all the drugs examined here.

The gram-positive cocci were the most sensitive with *Str. viridans* the most and *Staph. aureus* the least sensitive of the group. The gram-positive anaerobic bacilli were much more resistant than the cocci, but on the whole were more sensitive than the gram-negative bacilli, excepting the dysentery bacteria. *Cl. oedematiens* was the most and *Cl. welchii* the least sensitive of the anaerobes.

The three most sensitive of the gram-negative bacilli were *B. shigae*, pasteurella and *B. flexneri*. *B. typhosum* was the most sensitive and *B. typhi-murium* the most resistant

Table 4. *Limiting inhibitory dilutions of drugs in thousands against dysentery and other gram-negative organisms in broth and 50% serum broth*

Drug	*B. shigae*		*B. flexneri*		*B. sonnei*		*Pasteurella*		*B. proteus*		*B. pyocyaneus*	
	Broth	Serum	Broth	Serum	Broth	Serum	Broth	Serum	Broth	Serum	Broth	Serum
6-Diamine	0·1	0·3	—	—	—	—	—	—	—	—	—	—
14- ,,	20	200	5	60	5	50	60	50	15	20	<10	10
16- ,,	100	80	50	40	25	20	150	30	15	10	<10	5
18- ,,	100	20	150	15	100	10	150	20	<20	<5	<20	<5
8-Diamidine	15	200	<5	<5	0·8	5	2	4	<5	<5	<5	<5
9- ,,	40	400	<5	10	2	7	4	5	<5	<5	<5	<5
10- ,,	100	1000	10	50	4	20	10	5	<5	<5	<5	5
11- ,,	350	1000	15	100	15	40	20	20	<5	7	<5	15
12- ,,	700	2000	40	200	20	80	75	40	<5	15	5	20
13- ,,	700	3000	75	100	100	100	150	50	5	20	5	20
14- ,,	300	2000	100	70	100	100	200	80	10	20	10	7
16- ,,	100	100	75	20	100	20	150	80	20	7	15	<5
Stilbene-diamidine	500	800	35	80	<20	50	80	80	<20	20	<20	7
5-Diguanidine	0·2	1·5	0·15	0·5	0·05	0·6	0·5	1·5	—	—	—	—
6- ,,	0·5	2	0·2	1	0·1	0·8	2	5	—	—	—	—
8- ,,	15	100	3	10	1·8	10	5	10	<5	<5	<5	<5
10- ,,	500	1500	40	120	15	40	60	40	<5	7	<5	10
12- ,,	2000	4000	160	150	75	150	150	50	5	15	5	15
14- ,,	3000	5000	500	150	500	200	700	300	20	15	40	15
16- ,,	700	1000	500	100	350	50	700	200	100	5	75	7
18- ,,	500	500	400	80	350	40	350	150	75	<10	50	<10
4-Di-*iso*thiourea	10	20	10	7	30	5	50	<5	10	5	<10	<5
6- ,,	500	5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
8- ,,	150	10	100	5	40	<5	100	<10	10	<10	<10	<10
11- ,,	40	60	40	60	20	40	100	80	<5	7	10	5
12- ,,	30	60	30	20	7	20	100	30	<5	10	5	7
16- ,,	75	20	50	20	20	20	200	50	10	15	10	7
8-Di-quaternary base	0·1	0·3	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
14- ,,	5	50	<5	10	<5	<5	<5	<5	<5	<5	<5	<5
9-Amine	5	20	5	7	5	5	15	7	<5	5	<5	<5
12- ,,	100	20	100	7	100	5	200	30	50	<5	15	<5
14- ,,	100	10	100	<10	20	<10	350	<10	15	<10	10	<10
16- ,,	<10	<20	<10	<20	<10	<20	150	<20	<10	<20	<10	<20
18- ,,	<20	<20	<20	<20	<20	<20	<20	<20	<10	<20	<10	<20
15-Amidine	75	<20	100	<20	50	<20	75	<20	50	<20	<10	<20
17- ,,	<20	<10	<20	<10	<20	<10	50	<10	<20	<20	<20	<20
9-Guanidine	75	50	60	40	30	20	100	50	20	5	20	5
14- ,,	300	<10	200	<10	100	<10	150	20	30	<10	<10	<10
16- ,,	<20	<10	<20	<20	<20	<10	100	<10	<20	<10	<20	<10
18- ,,	<20	<10	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
9-Quaternary base	10	20	5	<5	<5	<5	30	30	<5	<5	<5	<20
18- ,,	35	<0·5	10	<5	5	<5	100	<5	10	<5	<5	<5

of the colon-typhoid group. *B. proteus* and *B. pyocyaneus* were the most resistant of all the organisms tested.

Closely related pairs of organisms were similar in behaviour, for example *B. typhosum* and *B. enteritidis*, Dublin (Colon-typhoid sub-group D), and *B. paratyphosum* B, and *B. typhi-murium* (sub-group B).

A few of the drugs differ markedly in their actions towards gram-positive and gram-negative bacteria. For example, the 16- and 18-amines and guanidines were very active against gram-positive, but were inactive against gram-negative bacteria, except *B. cholerae* and pasteurella. These last two were similar also in being much more sensitive than the other gram-negative bacilli to 18-quaternary base. The diamines were much more active against gram-positive than against gram-negative bacteria, both in actual titres and in comparison with the other drugs. The dysentery strains were unusually resistant to the diamines and di-*iso*thioureas, but were sensitive to the more powerfully basic diguanidines and quaternary salts.

Effect of chain length. The activity in all the series of drugs (except the di-*iso*thioureas) increased with the length of the carbon chain up to $n=14$ to 18 for the disubstituted compounds and up to $n=12$ to 16 for the monosubstituted compounds. The diamine and diamidine series had not reached maximal activity against gram-positive organisms by $n=18$, but with this exception the series had reached a stationary or a declining phase before this point. In the disubstituted series the fall in activity with increasing chain length beyond the optimal was gradual, but in the monosubstituted series the fall was much sharper, especially against gram-negative bacteria. Thus the drugs of the monosubstituted series with $n=16$ or more were inactive against practically all the gram-negative bacteria, although they were potent against the gram-positives.

The diguanidines reached their maximum activity at a shorter chain length than the diamines or diamidines, but the reverse held in the monosubstituted series.

Stilbene-diamidine, which includes two benzene rings, had approximately the activity of the $n=12$ open chain compound.

The rates of change of activity varied greatly in the different series. There was sometimes a 20- to 30-fold increase of titre for an addition of $2n$, as for example from 12- to 14-diguanidine against the cocci, but in general the changes were more gradual.

Drugs of chain length lower than $n=8$ had very slight activity except in the di-*iso*thiourea series, the activity of which increased and decreased sharply as the series was ascended.

Effect of the terminal group. The effects of mono- or di-substitution, and of changes in the end groups can be seen by comparing compounds of the same chain length. The disubstituted guanidines were far more active than the monosubstituted against all bacteria. In the other series the monosubstituted compounds had, on the whole, a slight advantage, especially against the anaerobes and the colon-typhoid group. The monosubstituted series reached maximum activity at a shorter chain length than the disubstituted, which gave the higher members of the latter a great advantage, especially against gram-negative bacteria.

The di-*iso*thioureas appeared to be by far the most active at chain lengths of 4 and 6, while the diguanidines were, with few exceptions, the most active from $n=10$ to $n=18$. The advantage of the diguanidines became less as the chain length increased.

The diamines were more active than the diamidines and much more active than the quaternary bases. The activities in the diamine and diamidine series started to decrease at a shorter chain length against gram-negative than against gram-positive bacteria, but the opposite was true in the diguanidine series. The guanidines were the most active in the monosubstituted series, but had not the overwhelming superiority of the diguanidines. The comparison between the strongly basic quaternary bases and the weakly basic amines was incomplete, and a more complete comparison would be interesting. The 8-di-quaternary salt was similar in activity to the 6-diamine. The 9 mono- and the 14-di-quaternary salts were much less active, and the 18-mono- more active than the corresponding amines.

Effect of serum

Table 5 shows how the activities of the drugs against different classes of bacteria were affected by the presence of serum. It has the disadvantage that bacteria behaving differently have been grouped together, but the influence of serum on individual strains can be seen in Tables 1–4.

Besides its direct effect on the drug, serum affects the titres by obscuring small amounts of growth in the opalescent serum, and by its effect on the growth-rate of some bacteria.

Disubstituted drugs. Serum caused an increase in the activity of the lowest members of the disubstituted series, except in the di-*iso*thiourea group and against *Str. pyogenes*. As the chain length increased, this advantage steadily decreased until at $n=16$ or 18,

Table 5. *Activity of drugs in 50% serum expressed as a percentage of the activity in broth*

Group and '*n*'		Cocci	Anaerobes	Dysentery	Rest of gram-negative
Diamine	-6	40	—	300	—
	-14	220	280	900	220
	-16	13	80	80	40
	-18	5	10	17	14
Diamidines	-8	220	—	1000	600
	-9	190	—	650	600
	-10	150	—	650	550
	-11	130	—	550	270
	-12	400	25	400	230
	-13	320	36	210	230
	-14	250	18	310	70
	-16	65	14	50	30
Stilbene		100	—	200	200
Diguanidines	-5	300	—	800	500
	-6	200	—	600	800
	-8	185	—	500	300
	-10	140	110	300	220
	-12	130	70	150	200
	-14	20	17	70	45
	-16	40	15	60	11
	-18	15	9	40	15
Di-*iso*thioureas	-4	10	12	90	30
	-6	200	—	? 500	? 1000
	-8	20	6	8	5
	-11	40	60	170	100
	-12	60	50	180	180
	-16	20	25	55	80
Di-quaternary	-8	—	—	—	—
	-14	—	—	—	900
Amines	-9	250	150	200	150
	-12	5	11	11	10
	-14	4	5	—	3
	-16	5	4	—	—
	-18	11	—	—	—
Amidines	-15	20	<20	<20	<20
	-17	—	—	—	—
Guanidines	-9	100	25	67	40
	-14	15	10	3	<10
	-16	8	—	—	—
	-18	17	—	—	—
Quaternary salt	-9	500	—	200	150
	-18	11	6	1	< 5

the drug had only a fraction of its activity in broth (Table 5). Thus maximal activity occurred at a shorter chain length in serum than in broth.

Against staphylococcus, *B. shigae* and *B. typhosum* the ratios of serum to broth activities (S/B) varied from 5 to 13 for the shortest chains, falling steadily to unity or less for the longest chains. With *Str. viridans*, pneumococcus, pasteurella and the anaerobes, serum interfered more, so that the values were seldom over unity even for the short chains, and fell further as the chain lengthened. Other organisms were intermediate in their behaviour. The activity against *Str. viridans* was the most depressed of any, possibly owing to the growth stimulation produced by serum on broth cultures of this organism. With *Str. pyogenes*, serum weakened drugs with chain lengths below $n=10$ and above $n=14$, and strengthened the middle members. In the di-*iso*thiourea group the least active compounds were strengthened, and the most active weakened, so that in serum there was a resultant increase of activity with chain length.

Serum activated the diamidine series against *Str. pyogenes* and staphylococcus. On the other hand the diamidines were very much weakened against the anaerobes and the di-*iso*thioureas against staphylococcus. The great sensitivity of *B. shigae* to the diamidines and diguanidines in serum is striking.

Monosubstituted drugs. These were much more strongly inhibited by serum than the corresponding members of the disubstituted series, and the point of serious interference occurred at a shorter chain length. Only in the 9-amine and 9-quaternary salt was an increase in activity in serum observed, and by $n=12$ the activity was only about 10% of the activity in broth. Consequently the higher mono-amines, -amidines and -guanidines were inactive against all organisms except some cocci. 9-Amine and 9-guanidine were equally active against gram-positive, but the guanidine was by far the more potent against gram-negative bacteria.

Titres in serum. It will be seen from Tables 1–4 that the diguanidines came out best of the disubstituted series against all bacteria, especially the gram-negative ones. The 14- and 16-diamines and 16-diamidine also were prominently active. The higher diamines were the most active against *Str. viridans* and anaerobes, the higher diamidines against *Str. pyogenes* and *Staph. aureus*, and the higher diamidines and diguanidines against dysentery.

In the monosubstituted series, the guanidines were best against *Str. pyogenes*, *Staph. aureus* and gram-negative organisms. No compound with chain length above $n=14$ was active against any of these last. The higher amidines were best against *Str. viridans*, and the amines against the anaerobes. 9-Quaternary base was unusually active against *Str. pyogenes*.

Although the activities of some drugs were increased 10-fold against *B. shigae* and *Str. pyogenes*, serum interfered most seriously with the most active drugs, and this limited the peak activity of any series. For example, the activity of 4-di-*iso*thiourea against *Str. viridans* was reduced from 1 in 1,000,000 to 1 in 10,000, and that of 12-amine against pneumococcus from 1 in 7,000,000 to 1 in 10,000.

Serum caused some change in the order of sensitivity of the bacteria. *Str. pyogenes* became the most and pneumococcus the least sensitive of the cocci. *Cl. oedematiens* remained the most sensitive of the anaerobes. *B. shigae* was by far the most sensitive of the gram-negative organisms, and *B. typhosum* next.

Strain variation

The above work had been carried out with one strain only of each species and it was possible that considerable strain variations might occur. To get some idea of possible strain variation, the experiments with some of the drugs were carried out on the following 25 strains of dysentery:

2 of *B. shigae*, 1 of *B. sonnei*, 1 of *B. ambigua* and the following Flexner strains: 3 of V, 2 of W, 1 of X, 2 of Z, 2 of 103, 3 of 170, 2 of 274, 1 of 119, 2 of 288, 2 of 88 and 1 of D.

The variation in the inhibitory titres for the Flexner strains can be seen from Table 6. They are for the most part inconsiderable, especially as the results were not checked as thoroughly as in the main series.

Table 6. *Titres of drugs against dysentery strains*

Drug	Range of titres in broth	Range of titres in serum-broth
18-Diamine	100,000–200,000	20,000
13-Diamidine	50,000–100,000	40,000–100,000
14-Diamidine	50,000–100,000	20,000–100,000
16-Diamidine	75,000–100,000	20,000
14-Diguanidine	200,000–1,000,000	100,000–500,000
16-Diguanidine	200,000–500,000	50,000–200,000
18-Diguanidine	300,000–500,000	50,000–100,000
8-Di-*iso*thiourea	50,000–100,000	20,000
12-Amine	100,000	20,000
14-Amine	100,000–200,000	20,000
15-Amidine	100,000	20,000
14-Guanidine	100,000–200,000	20,000

Sulphanilamide-resistant strains. Three group A streptococcus pyogenes strains, 'Mead', 'Kingsbury', and 'Atkinson', resistant to sulphanilamide, and two other sensitive strains, 'Pickering' and 'Wood' were tested. They were recently isolated from wounds and were kindly supplied by Dr Colebrook. The results (Table 7) show that resistance to sulphanilamide does not affect the sensitivity to the present series of drugs. The titres were not determined as exhaustively as in the main series and the differences were probably not as great as indicated in Table 7.

Table 7. *Titres of sulphanilamide-sensitive and -resistant strains*

Strain	14-Diamidine	14-Diguanidine	14-Guanidine	12-Amine
'Sensitive'				
Richards	200,000	2,000,000	500,000	700,000
Pickering	100,000	1,000,000	—	300,000
Wood	500,000	1,500,000	—	—
'Resistant'				
Mead	400,000	4,000,000	800,000	1,000,000
Kingsbury	100,000	1,500,000	500,000	1,000,000
Atkinson	400,000	2,000,000	800,000	800,000

Effect of repeated subculture on sensitivity

It was noticed that when there was a difference in sensitivity of strains of dysentery organisms, the old stock strains were usually the most resistant, and recently isolated strains the most sensitive. Six recently isolated strains of *B. Flexneri* were subcultured daily in broth thirty times. The titres of twelve of the drugs for the passaged strains were compared with the original titres. The titres for each drug against all the strains were averaged and it was found that the passaged strains were inhibited by different drugs at 1·2–5·0 times the concentration as compared with the original strains. An average of all the results showed the passage strains to be twice as resistant as formerly.

Therapeutic effect

King *et al.* [1938] determined the toxicities of the drugs, which have a mouse-tolerated dose of 1–4 mg. for the lowest members and 0·1–0·5 mg. for the higher members. 18-Amine and 18-guanidine are less toxic than the lower members.

Multiplying the 50% serum titres of the drugs by the mouse-tolerated doses gives a measure of their probable therapeutic efficiencies, so far as these can be deduced from *in vitro* testing. Compounds giving high values are 14-diamine, 14-diamidine and 14-guanidine. 11-Di-*iso*thiourea is the best of its group, especially against gram-negative bacteria; 9- and 12-amines are the best of the monosubstituted compounds.

Dr F. Hawking kindly tested some of these compounds in mice infected intraperitoneally with *Str. pyogenes*, 'Richards'. The drugs were given subcutaneously twice a day for 3 days, and once a day for the next 3 days, in the doses per g. of mouse given below. No significant therapeutic effect was obtained.

	mg.		mg.
13- and 14-diamidines	0·005	9-Amidine	0·05
Stilbene-diamidine	0·01	18-Guanidine	0·05
14-Diamine	0·02	9-Quaternary salt	0·025
14-Diguanidine	0·001		

Discussion

In vitro antibacterial power is the most important single factor in the activity of the chemotherapeutic drugs in use to-day, and the *in vitro* technique, by its speed and precision, is of use in investigating new drugs. It was hoped, by using a large range of bacteria, and several series of related drugs, to discover some of the underlying principles

of drug specificity. This, unfortunately, has not been possible, but the antibacterial action of the present series is worth recording.

The chief feature is the increase of activity with chain length up to a certain point, followed by a decrease beyond that point. The exception to this rule is the curious alternation of compounds with high and low activity in the di-*iso*thioureas, and the potency of the lowest member. This series differs from the others in having sulphur atoms in the carbon chain. Baker, Harrison & Miller [1941] found that the straight-chain alkyl sulphates had maximum antibacterial activity at 12–16 carbon atoms.

The increase of activity with chain length suggests that surface-active properties may be responsible for the activity of these compounds and that they may act by denaturing some essential protein constituent [Anson, 1939]. It would be interesting to determine the effect of branched chain derivatives, or those having more than two active centres, since alkyl sulphates of this type are very powerfully surface-active [Caryl & Ericks, 1939].

Being basic, the drugs may combine with a bacterial protein, e.g. a nucleoprotein, and render it inert; this is supported by the fact that the most active compounds tend to be the most toxic and the most inhibited by serum. Serum may act by affecting the adsorption of the drug by the bacteria.

The bacteriostatic titres are much lower than the trypanocidal titres, but the variations of activity with chain length are similar in both cases. It is probable that the same mechanism is responsible for both actions of the drugs, and that these may be general protoplasmic poisons.

Taking the results as a whole, the differences in activity caused by interchange of the end groups are not large. The chain length is usually far more important than the end group. The basicity of these groups presumably increases in the following order—di-*iso*thioureas, amines, amidines, guanidines and quaternary ammonium salts. It does not appear that activity depends on a particular balance between the chain length and the basicity of the end groups. A more complete comparison between the amines and the quaternary salts would be interesting in view of the great difference in basicity between them.

The disubstituted drugs are on the whole more satisfactory than the monosubstituted. The higher members are more active at equal chain length and are far less inhibited by serum, but are more toxic.

One generalization which can be made is that the less strongly basic amines have a relatively stronger action on the gram-positive bacteria while the strongly basic guanidines and quaternary bases have a relatively stronger action on the gram-negative organisms. This is very marked in the dysentery group, against which the diamines and di-*iso*thioureas are weakly active, and the corresponding amidines, guanidines and quaternary bases relatively stronger. This holds for both the mono- and di-substituted series, and it would appear that increasing the basicity of the end groups is likely to make the compound more active against gram-negative and relatively less active against gram-positive organisms.

If the bacteria are arranged according to sensitivity to the drugs, the order in which they fall is broadly similar to that for the sulphonamides, i.e. those of greatest metabolic activity are the most resistant. Small differences occur, e.g. *Cl. oedematiens* is the most sensitive of the anaerobes to the present series, whereas it is the most resistant to the sulphonamides. The present series too are active in much higher dilution, and are powerfully bacteriostatic in media (broth and serum-broth) in which the sulphonamides have little action. Although the activity of the drugs in broth is considerable, their high toxicity and the interference of serum with the most active are against their chemotherapeutic use, and Dr Hawking's tests show them to be inactive against *Str. pyogenes*. In this they resemble gramicidin [Dubos & Hotchkiss, 1941] which is active in the body

only when applied locally. White, Bratton, Litchfield & Marshall [1941] have found that many substances active *in vitro* are without therapeutic effect. The drugs might be useful for local application against sulphonamide-resistant organisms, and the very sparing solubility of the free bases might confer advantages in the treatment of enteric infections.

Summary

The antibacterial activity of straight-chain ω-mono- and di-amines, amidines, guanidines and quaternary bases in broth increases with chain length up to a maximum and then decreases. The corresponding changes in the di-*iso*thioureas are irregular.

Gram-positive organisms are on the whole more sensitive than gram-negative, and the bacteria fall roughly in the same order for related drugs.

Serum usually causes an increase in the activity of the shorter-chain and a decrease in that of the longer-chain drugs. This effect, however, varies with the bacteria concerned, and it causes maximum activity in a series to occur at a shorter chain length than in broth.

The drugs are active against sulphanilamide-resistant haemolytic streptococci, but have no therapeutic action when applied systemically.

My thanks are due to Dr Harold King for supplying the drugs, and to Dr Colebrook for the strains, and to both for advice generously given. I am also indebted to Dr A. N. Drury for a supply of human serum, and to Dr F. Hawking for carrying out animal tests.

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65. The Acetone-Soluble Lipid of the Atheromatous Aorta*

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Aschoff [1906] first drew attention to the high content of cholesterol in atheromatous aortas. Since that time many quantitative micro-analyses have been performed on the lipids of whole aortas showing varying degrees of atheroma. Schoenheimer [1926; 1928] concluded that the total extractable lipid of the aorta increased with the age of the individual and the severity of atherosclerosis. Furthermore, in the early stages the relative amounts of phospholipid and cholesteryl esters increased with increasing total extractable lipid. A stage was reached, however, at which the percentage of phospholipid and cholesteryl ester in the total lipid became constant. He explained these results on the basis of infiltration and deposition of a lipid mixture elaborated elsewhere. These and other early investigations have been reviewed by Wells [1933].

Later studies [Meeker & Jobling, 1934; Zeek, 1936] have dealt with the changing ratios between total cholesterol, free cholesterol and phospholipids in isolated plaques classified according to the degree of atheroma. The results of these more extensive analytical investigations were at variance with those of Schoenheimer. They indicated that the values of the ratio free cholesterol/bound cholesterol in early plaques were lower than those for normal intimal tissues, while in the advanced lesions they were normal or above normal. Total cholesterol, cholesteryl esters, fatty acids and lecithin were found to increase with the progress of atheroma. Weinhouse & Hirsch [1940] and Page [1941] determined free cholesterol, cholesteryl esters and phospholipids (neutral fat by difference) in the lipid mixtures of normal human plasma and atheromatous plaques, and drew attention to the quantitative similarity in the compositions of these lipids.

Comparatively little work has been done on the isolation or identification of the individual constituents of the various fractions of the lipid from atheromatous aortas. Schoenheimer [1928] isolated free cholesterol, the cholesteryl esters of oleic, palmitic and stearic acids, and a galactoside, by fractional crystallization of the acetone, light petroleum (l.p.) and alcoholic extracts of the adventitia-free atheromatous aortas. He also found indications of the presence of 'oxycholesterol' and fatty acids having more than one double bond, but these were not identified. He failed to find glycerides in the mixture. Schoenheimer, von Behring & Hummel [1930] investigated the saturated sterol content of the lipids from various animal sources. Of the many tissues examined, atheromatous aortas yielded unsaponifiable lipid having the highest values for saturated sterol content (5·1–5·3% of the total sterol). The suggestion was made that such saturated sterols might have been formed by intermediary hydrogenation of cholesterol. However, it is worthy of note that Gardner & Gainsborough [1934] found Schoenheimer's method for separating saturated from unsaturated sterols unsatisfactory from a quantitative point of view. They were not successful in obtaining the same high yields of β-cholestanol from gall-stone cholesterol as were reported by Schoenheimer.

The results of the quantitative studies mentioned above suggest that the plasma is the source of the lipids deposited in the intima. To test this suggestion, further identification of the fatty acids and the substances with which they are combined in the original fat will be required. The present investigation was undertaken to ascertain the nature of the

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individual components of the 'phospholipid-free'* fraction of the fat extracted from atheromatous intimas, in order that this might be compared with similar lipid fractions from other sources in the body, particularly the plasma.

Experimental

Preparation and extraction of the intimal tissue

Thirty-three complete aortas and parts of six others, all showing atheromatous plaques, were collected at autopsy. Immediately after removal from the cadaver each aorta was slit up the anterior side and the adventitial tissue was stripped off, care being taken to keep the intima uncontaminated by fatty substances from other tissues. The remaining media and intimal tissue were dropped into 30–40% ethanol to prevent decomposition during the 1–2 days which elapsed before the tissues became available for further treatment. On removal from the dilute alcohol the specimens were pinned on a board with the intimal surface down, while the media was stripped off as cleanly as possible. The remaining intima was ground finely in a food-chopper while still moist, regrinding being carried out if necessary. The minced material was spread out in evaporating dishes in a vacuum desiccator over anhydrous $CaCl_2$. This procedure removed most of the water in about 2 days. On being reground when dry the tissue was reduced to a fine powder suitable for extraction in the Soxhlet apparatus. The extraction was made with ether which had been freed from peroxide by distillation over $SnCl_2$. After removal of the ether from the extract by distillation under reduced pressure, and of moisture by subsequent distillation of benzene from the residue under reduced pressure, the lipid mixture remaining was transferred in l.p. solution from the distillation flask to a weighed round-bottom flask. The solvent was then removed by vacuum distillation and the residue weighed. The 39 aortas yielded 143 g. of dry, fat-free intimal tissue and 35·58 g. of ether-soluble lipid.

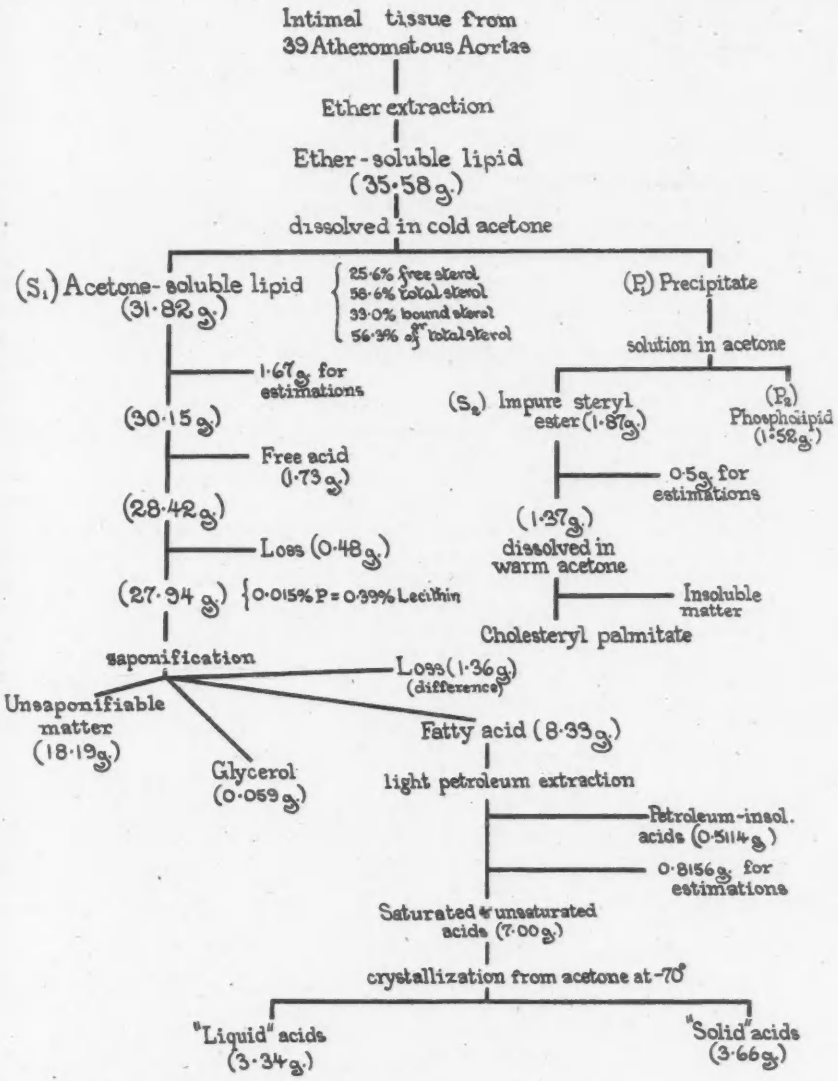
In order to remove the bulk of the phospholipid, the lipid mixture (35·58 g.) was taken up in 360 ml. pure, dry acetone and the mixture allowed to stand overnight at a temperature ranging between 5° and 9°. The acetone solution was filtered and the precipitate (P_1) (see scheme) well washed with ice-cold acetone. The combined washings and filtrate (S_1) were set aside. The precipitate (P_1) dissolved in l.p. was added to a small amount of cold acetone with vigorous stirring. This procedure brought about the precipitation of 1·52 g. of phospholipid (P_2) which was not further investigated. Evaporation of the filtrate (S_2) left a residue weighing 1·87 g. which proved to be steryl ester.

Acetone-insoluble steryl ester

The mixture (S_2) did not contain free cholesterol since it gave no digitonide. To determine its nature 400 mg. were saponified by refluxing for 8 hr. with 4% KOH in methanol and enough benzene to keep the lipid in solution. The unsaponifiable matter was extracted with l.p. in a continuous extractor. After removal of petroleum the residue was washed into a 50 ml. volumetric flask with 95% ethanol and diluted to volume. To 10 ml. in a 100 ml. Erlenmeyer flask 30 ml. of 1% digitonin in 90% ethanol were added in a thin stream while the reaction mixture was kept at 70°. After standing for 48 hr. at room temperature the digitonide was filtered on a sintered glass crucible, washed with 90% ethanol, ethyl ether and finally l.p. The precipitate, dried at 90° and cooled in a desiccator, weighed 0·1873 g., representing 0·0456 g. sterol (calculated as cholesterol). Therefore the original material (1·87 g.) contained 1·065 g. sterol equivalent to 1·723 g. (92·1%) steryl ester (calculated as cholesteryl palmitate).

* The acetone-soluble lipid is referred to as phospholipid-free fat although it contained small amounts of phospholipid.

The remainder (40 ml.) of the alcoholic solution of the unsaponifiable matter was concentrated and decolorized with activated carbon. The residue after recrystallization from dilute alcohol and from l.p. melted at 148·4° (corr.); M.P. of cholesterol 148°, 148·5°



[Sobotka, 1938]. Acetylation with acetic anhydride in the presence of anhydrous sodium acetate and recrystallization of the resulting product from dilute alcohol, yielded a compound melting at 114·4°; M.P. of cholesteryl acetate 115° [Page & Rudy, 1930].

A part of the remaining impure ester was used to characterize the fatty acid constituents. It was redissolved in a small volume of warm acetone, filtered from a small quantity of insoluble matter and taken to dryness. The residue was then saponified, extracted with l.p. to remove the unsaponifiable matter, acidified and extracted with l.p. to yield fatty acids having sap. equiv. 257·5, 258 (palmitic, 256·3). A small quantity of this fatty acid treated with activated carbon, recrystallized from dilute alcohol and dried over P_2O_5 melted at 62° (corr.); M.P. of palmitic acid is 62·6°. The acetone-insoluble ester fraction was therefore almost pure cholesteryl palmitate.

The acetone-soluble lipid fraction

Removal of the solvent from the acetone liquor (S_1) left a residue of acetone-soluble lipid weighing 31·82 g. 1·67 g. were reserved for analysis. One portion weighing 0·663 g. yielded directly 0·699 g. sterol digitonide, representing 0·170 g. sterol (calculated as cholesterol). Therefore the acetone-soluble lipid contained 25·6% free sterol. Two other portions weighing 0·5435 and 0·1199 g. were saponified, the unsaponifiable matter was extracted quantitatively and an excess of digitonin was added in the usual manner. The yields of sterol digitonide were 1·309 and 0·2892 g. respectively, equivalent to 0·3183 and 0·0703 g. cholesterol or 58·6% of the total lipid. The acetone-soluble lipid contained therefore 25·6% free sterol, 58·6% total sterol and by difference 33·0% combined sterol.

The remainder of the 1·67 g. sample was dissolved in ether and washed with aqueous Na_2CO_3. After removal of the ether the neutral lipid mixture contained 0·015% P, the equivalent of 0·39% phospholipid calculated as lecithin.

The total sterol in the phospholipid-free lipid (33·54 g.) may be calculated by summation of the sterol contents of fractions S_1 and S_2. In fraction S_2 containing 1·723 g. cholesteryl palmitate there were 1·065 g. bound sterol. Adding this to the values found for S_1 the total sterol content of the phospholipid-free fat becomes 19·72 g. and the free sterol 8·15 g. In other words the phospholipid-free lipid contains 58·8% total sterol and 24·3% free sterol (by difference bound sterol amounts to 34·5%).

The acetone-soluble lipid mixture (30·15 g.) was dissolved in 500 ml. peroxide-free ether and washed with 3 l. 1% aqueous Na_2CO_3 to remove acidic substances. Ether extraction of the acidified aqueous solution yielded 1·73 g. acidic material of very high equivalent weight (491) and containing 3·6% P. The nature of this material has not been determined although its high equivalent weight and P content suggest that it might contain phosphatidic acids. After removal of the solvent from the ether phase the neutral fraction weighed 27·94 g.; therefore the washing process was attended by a loss of 0·48 g.

The neutral lipid mixture (27·94 g.) was transferred with 25 ml. benzene into 140 ml. 6% methanolic KOH in a round-bottom flask. The mixture was refluxed for 3 hr. in a stream of O_2-free N_2. After cooling, the saponification mixture was poured into 700 ml. 30% ethanol and extracted five times with 200 ml. portions of peroxide-free ether, sufficient time being allowed after each shaking for sharp separation of the two layers. The ether solution was then washed once with 500 ml. and a second time with 250 ml. of 20% ethanol containing 0·5 g. KOH. The dilute alcoholic solutions were combined and set aside for the removal of the fatty acids.

The unsaponifiable matter from the acetone-soluble fraction

On removal of the solvent from the ether solution there remained 18·19 g. unsaponifiable matter, which is 65·1% of the neutral acetone-soluble lipid.

Three samples of the unsaponifiable matter (dried over P_2O_5 *in vacuo*) weighing 0·8034, 0·8224 and 0·2119 g. gave digitonides weighing respectively 3·054, 3·132 and 0·8041 g. These represent sterol percentages of 92·4, 92·6 and 92·3 (calculated as cholesterol), thus indicating that the unsaponifiable matter contained 92·4% (av.) or 16·81 g. of sterol.

Assuming that the losses were mechanical and therefore proportionally distributed between the components, it may be calculated from the data shown in the scheme that the amount of sterol to be expected at this point was 17·39 g. The amount of sterol lost during saponification and subsequent extraction of the products of hydrolysis was therefore 0·58 g.

The saturated sterol content of the unsaponifiable matter precipitated as digitonide was investigated by the method of Schoenheimer [1930]. The values found (11·7 and 12·9%) were rather more than double those reported by Schoenheimer, v. Behring & Hummel [1930] (5·1–5·3%) for the sterol mixture obtained from atheromatous aortas.

Further work is being done on the saturated sterols and their distribution amongst the components of atheromatous fat.

The total fatty acids from the acetone-soluble fraction

The dilute soap solution remaining after extraction of the unsaponifiable matter was acidified to Congo red with dilute HCl. This was followed by several extractions with peroxide-free ether to remove the free fatty acids. The aqueous alcoholic solution was set aside to be examined for glycerol content. The solvent was removed from the ethereal extract by distillation under reduced pressure and the residue dried by distilling benzene from it. A yield of 8·33 g. mixed fatty acids was thus obtained.

100 mg. of this fatty acid mixture dissolved in 25 ml. alcohol-ether mixture took 3·58 ml. of 0·0993*N* NaOH for neutralization, indicating equiv. wt. 281·3. The remainder of the acid mixture was extracted twice with small volumes of l.p. in which 0·5144 g. was insoluble. The petroleum-insoluble fatty acid had I.V. 58·5 (by the micro-method of Yasuda [1931]), but attempts to recrystallize its Pb and Ba salts were unsuccessful. After removal of the petroleum from the filtered extract, the residue had I.V. 104.

In view of the technical difficulties of separating such a small quantity of mixed fatty acids into 'liquid' and 'solid' fractions by the lead salt and ether or alcohol processes and the desirability of keeping the concentration of oleic acid in the unsaturated fraction low, the mixed fatty acids (7·00 g.) were crystallized from 300 ml. pure dry acetone at $-70°$ in a special recrystallization and filtration chamber which was cooled with alcohol and solid CO_2. This procedure yielded 3·66 g. of a solid fraction having I.V. 74. On removal of the acetone from the filtrate the residual liquid acid (3·34 g.) had I.V. 136.

The liquid fraction of the fatty acids

Repeated bromination and preliminary fractionation of the insoluble brominated acids. 3·137 g. of the liquid fraction of the fatty acids dissolved in 32 ml. pure anhydrous ether were brominated at $-10°$ by the addition of 1·7 g. dry Br over a period of 60 min. After standing 5 hr. at about $-15°$ the precipitate was filtered off in a sintered glass crucible and washed five times with 5 ml. portions of cold ether. This precipitate of ether-insoluble polybromide, dried at 80° for 3 hr., weighed 0·4165 g. It was reserved for later examination.

The ethereal filtrate was shaken with aqueous sodium thiosulphate to remove the excess of Br. After removal of the ether *in vacuo*, benzene was distilled from the residue under reduced pressure to remove moisture. Extraction of the residue with two 100 ml. portions of boiling l.p. left 0·504 g. dark-coloured insoluble gum.

The petroleum extract, on concentration to 100 ml. and standing overnight in a refrigerator, deposited a precipitate which when filtered off, washed with l.p. and dried, weighed 0·972 g. The filtrate and washings were concentrated and allowed to stand in the refrigerator for several weeks, when a further 0·389 g. of the petroleum-insoluble material precipitated. Thus the first bromination of the 'liquid' fraction of the fatty acids yielded 1·361 g. of ether-soluble petroleum-insoluble brominated fatty acids.

The oily residue (3·356 g.) obtained by evaporation of the petroleum filtrate was mixed with the petroleum-insoluble gum (0·504 g.) and 3·605 g. of the mixture were debrominated in accordance with the directions of Rollet [1909]. The methyl esters thus obtained weighed 1·987 g. and had I.V. 112. Since the I.V. of methyl oleate is 85·6, not all of the acids having more than one double bond had been removed by bromination. The free fatty acids were recovered after cold saponification. This was carried out by allowing 1·974 g. of the ester mixture to stand overnight in 20 ml. 5% methanolic KOH. The saponification mixture was then poured into an equal volume of distilled water and extracted with l.p. to remove traces of ester which might have escaped hydrolysis.

The soap solution was acidified and extracted once with l.p. and once with benzene. Removal of the solvents *in vacuo* left a residue of fatty acids weighing 1·787 g. and with I.V. 119.

1·771 g. of the acid mixture were rebrominated in 20 ml. ether in the same way as before and yielded 0·0555 g. of the ether-insoluble brominated acid and 0·080 g. of the petroleum-insoluble brominated compound. Evaporation of the l.p. filtrate left a residue of soluble brominated acids. On debromination this residue yielded methyl esters having I.V. 94·2; consequently two brominations had failed to remove completely the unsaturated acids having more than one double bond. However, the amount of the highly unsaturated fatty acids which escaped precipitation as bromo-derivatives by virtue of the solubility of their β-forms was so small, that it was considered inadvisable to subject the remainder of the acid mixture to further bromination.

To determine whether any saturated acids had been carried over with the unsaturated fraction, 1·137 g. of the residual methyl ester were treated with hydroxylamine and sodium ethoxide in absolute alcohol, according to the method of Lewis [1926]. For this test, alcoholic hydroxylamine was prepared according to Lewis except that only the clear supernatant liquid pipetted off from the precipitate of NaCl was used. The reaction mixture on standing for 45 days at 10° gave no precipitate, and when concentrated and cooled to $-10°$ gave no evidence of the presence of insoluble hydroxamates. It was concluded that the liquid fraction contained no appreciable quantity of the saturated fatty acids. Furthermore, since the methyl esters, which appeared to contain only very small amounts of fatty acids having more than two double bonds, had I.V. 94·2 (94·0, 94·4), the mixture probably consisted of about 90% methyl oleate and 10% methyl linoleate.

Arachidonic acid. At various stages there had been collected, in all, 0·4720 g. ether-insoluble polybromide, and according to its bromine content (67·3%) this appeared to be almost all octabromo-arachidic acid. The ether-insoluble polybromide was extracted with pure hot dioxan until only a small amount (57·3 mg.) of residue remained. This had a decomposition temperature of 233° (uncorr.) and gave the following analytical figures: C, 25·0, 25·1; H, 3·49, 3·58; Br, 67·5, 67·3. $C_{20}H_{32}O_2Br_8$ requires: C, 25·5; H, 3·42; Br, 67·7; decomp. 240°.

Linoleic acid. The ether-soluble brominated fatty acid (1·441 g.) which crystallized from l.p. was redissolved in boiling petroleum (B.P. 80–100°), treated with activated carbon and filtered while hot. White needles separated from the petroleum on standing overnight in the refrigerator. A second recrystallization yielded a product having M.P. 114·3° (corr.) and a neutral equivalent of 595 (593, 596). The substance was therefore tetrabromostearic acid (M.P. 113–114°; mol. wt. 600) derived from linoleic acid.

By means of two complete bromination procedures, insoluble bromine derivatives of arachidonic and linoleic acids were isolated from 3·137 g. of the liquid fraction of the fatty acids, in quantities equivalent to 0·1583 g. of arachidonic acid and 0·6858 g. of linoleic acid (corrections for aliquots having been made). Therefore the liquid fraction of the fatty acid contained about 5% arachidonic acid, 22% linoleic acid (isolated as the so-called α-forms) and 73% oleic acid (including small amounts of the β-forms of arachidonic and linoleic acids).

The solid fraction of the fatty acids

The usual method of resolving a mixture of saturated fatty acids by fractional distillation of their methyl esters was not attempted since the mixture in hand was largely oleic acid and the amount of saturated acid present was very small.

Lewis [1926] has investigated the relative solubilities of the sodium salts of the corresponding hydroxamic acids of butyric, octoic, lauric, oleic, palmitic and stearic acids in alcohol. The solubilities of the C_{16} and C_{18} saturated acid derivatives were so slight that these could be quantitatively separated from the compounds of butyric, octoic and oleic acids. However, the solubility of the lauric acid compound was such that it was considered impossible to obtain a theoretical separation of this acid from palmitic or stearic acids under the conditions given for the other separation.

In view of Lewis's findings, the acids of the solid fraction were converted into the corresponding sodium hydroxamates and these were fractionally crystallized. This procedure served to resolve the mixture into simpler fractions whose approximate composition could be calculated from their neutral equivalents and iodine values.

Preparation and fractionation of the hydroxamic acid derivatives

3·634 g. of the solid fatty acid mixture were dissolved in 25 ml. pure anhydrous ether into which diazomethane was passed. After removal of the solvent, the residue of methyl esters was treated with hydroxylamine in the presence of sodium ethoxide and the reaction mixture was left in a flask carrying a $CaCl_2$ tube for 45 hr. at 9°. The precipitate that formed, when filtered off and suspended in dilute acetic acid, yielded 0·9710 g. of free hydroxamic acid (fraction 1). Repeated concentration of the mother liquors followed by cooling, filtration and suspension of the sodium salts in dilute acetic acid, yielded a second and a third fraction while a fourth fraction was obtained by the addition of dilute acetic acid and water to the mother liquor from fraction 3. Fraction 1 was recrystallized from 25 ml. methanol to give four subfractions, 1 (*a*), 1 (*b*), 1 (*c*) and 1 (*d*). The weights and melting points of all these fractions are recorded in Table 1. Each of them was subjected to acid hydrolysis which liberated fatty acids in the amounts and of the characteristics also shown in Table 1.

Table 1

	Hydroxamic acid		Free fatty acid					
						Composition		
Fraction	Wt., g.	M.P.	Wt., g.	Neutral equivalent	I.V.	g.	%	Fatty acid
1 (*a*)	0·2360	92–93°	0·4199	264·8	—	0·2914	69·4	Palmitic
1 (*b*)	0·2074	92°				0·1285	30·6	Stearic
1 (*c*)	0·1065	90°	0·1010	271·4	10·5	0·0464	45·9	Palmitic
						0·0428	42·4	Stearic
						0·0118	11·7	Oleic
1 (*d*)	0·4211	Oil	0·3996	278·0	66·9	0·0655	16·4	Palmitic
						0·0364	9·1	Stearic
						0·2977	74·5	Oleic
2	0·4152	Oil	0·3944	278·7	71·5	0·0592	15·0	Palmitic
						0·0213	5·4	Stearic
						0·3139	79·6	Oleic
3	1·423	Oil	1·352	280·0	81·2	0·1298	9·6	Palmitic
						1·222	90·4	Oleic
4	0·9327	Oil	0·8861	279·0	89·2	0·8666	97·8	Oleic + β-forms
						0·0177	2·0	Linoleic
						0·0018	0·2	Arachidonic

The fatty acids from sub-fractions 1 (*a*) and 1 (*b*) were pooled since they had practically the same properties, and the ratio of palmitic acid to stearic acid was calculated from the

equivalent weight. The composition of the acids derived from the remaining sub-fractions was calculated from the iodine values in conjunction with the corresponding equivalents. Similar calculations were applied to fractions 2, 3 and 4 (see Table 1).

Arachidonic and linoleic acids in the solid fraction. 0·510 g. of the derived fatty acid from fraction 4 of the hydroxamic acids was subjected to bromination, giving 2·7 mg. ether-insoluble polybromide representing 0·9 mg. arachidonic acid and 20·4 mg. petroleum-insoluble tetrabromide, the equivalent of 9·5 mg. linoleic acid. Therefore this fraction contained about 0·2% α-arachidonic acid, 2·0% α-linoleic acid and 97·8% oleic acid with small amounts of the β-forms of linoleic and arachidonic acids.

Summation of the results of these estimations indicated that the solid fraction (3·634 g.) of the fatty acids contained 0·5923 g. palmitic acid, 0·2290 g. stearic acid, 2·712 g. oleic acid plus β-acids, 0·0177 g. linoleic acid and 0·0018 g. arachidonic acid. The fractionation was attended by a loss of 0·080 g. The percentage composition of the solid fraction is given in Table 2.

The approximate composition of the total fatty acids

Table 2 gives also the composition of the mixed fatty acids (free of petroleum-insoluble acids) obtained by summation of the analytical data for the liquid and solid fractions. Adding the amounts of palmitic acid bound in the acetone-insoluble cholesteryl palmitate and petroleum-insoluble acids to the above figures gave the approximate distribution for the total bound fatty acid contained in the phospholipid-free fat (Table 2).

Table 2. *Compositions of fatty-acid fractions* (%)

	Liquid	Solid	Liquid + solid	Liquid + solid + petroleum-insoluble acids + palmitic acid from cholesteryl palmitate
Stearic acid	—	6·4	3·4	2·9
Palmitic acid	—	16·7	8·7	14·6
Oleic + β-acids	73	76·3	74·7	65·2
Linoleic acid	22	0·5	10·8	9·4
Arachidonic acid	5	0·1	2·4	2·1
Petroleum-insoluble acid	—	—	—	5·8

The glycerol fraction

Isolation and identification of glycerol.

The acidic aqueous-alcoholic solution remaining after extraction of the fatty acids from the saponification mixture was made slightly alkaline by the addition of Na_2CO_3 solution and then brought back to a slightly acidic reaction with acetic acid. The alcohol and water were removed by distillation under reduced pressure. When the residue was almost dry, 95% ethanol was added and refluxed to wash down the walls of the flask. After removal of the alcohol by distillation under reduced pressure, 25 ml. dioxan* were added to the salty residue. The bottom of the flask was heated on a steam bath. A small ball of glass wool was dropped into the flask and the dioxan solution was drawn through this with a pipette for transference to a small distillation flask. This procedure was repeated twice with smaller volumes of dioxan. On removal of the dioxan by distillation under reduced pressure, the small amount of residue in the flask was extracted with pure anhydrous ether to remove any traces of fatty substance that might have been left after extraction of the fatty acids. The residue was transferred in aqueous solution to a weighed test tube which was then placed over anhydrous $CaCl_2$ in a vacuum desiccator. The dry residue weighed 59 mg.

* The dioxan had been refluxed for 48 hr. with metallic Na and afterwards distilled.

To the residue in the test tube, 0·67 ml. benzoyl chloride and 6 ml. 10% NaOH were added. The tube was stoppered and shaken for 10–15 min. The solid benzoate which formed was removed, dissolved in 10 ml. 95% alcohol and treated with a small amount of active carbon. After filtration, water was added to the filtrate and this was then set aside to crystallize. The crystalline product so obtained when recrystallized from dilute ethanol had M.P. 71°, and an authentic specimen of glyceryl tribenzoate prepared in the same manner melted at 72·5°. The accepted M.P. of glyceryl tribenzoate is 71–72°; the value of 76° quoted in some references is for a metastable form rarely encountered [Huntress & Mulliken, 1941]. A mixture of the product and the authentic glycerol tribenzoate melted at 72° while the mixed M.P. with authentic ethylene glycol dibenzoate (M.P. 71·5–72°) was 45–48°.

Calculation of the glyceride content of the acetone-soluble lipid

The glyceride content of the total phosphatide-free fat was calculated on the basis of the amount (0·059 g.) of glycerol isolated from the neutral fraction (28·42 g.) of the acetone-soluble lipid. Since the equivalent of the fatty acids was 281·3, the amount of glycerol isolated represented 0·565 g. of glyceride. Therefore the total acetone-soluble lipid (31·82 g.) contained 0·596 g. of glyceride and the original phosphatide-free fat (31·82 g. acetone-soluble lipid S_1+1·72 g. cholesteryl palmitate from S_2=33·54 g.) contained the same amount of glyceride or 1·8%.

The amount of bound fatty acid in combination with substances other than sterol was calculated in the following manner. The acetone-soluble lipid (30·15 g.) was found to contain 58·6% total sterol (i.e. 17·66 g.). During removal of the acidic material (1·73 g.) from the acetone-soluble lipid a loss of 0·48 g. was noted. The loss appeared to be purely mechanical and probably should be distributed proportionately between the fractions, so that the true weight of the neutral material would be 28·39 g. and would include 17·66 g. total sterol. The 27·94 g. taken for saponification should therefore contain 17·38 g. total sterol. Assuming that 56·3% of this is bound and that the average equivalent weight of the fatty acids combined with the sterol is 281·3, then 7·11 g. of fatty acid are combined with sterol. Since the total combined fatty acids found weighed 8·33 g., obviously 1·22 g. were bound by non-sterol alcohol.

On the assumption that all of this non-sterol alcohol was glycerol, the neutral material (27·94 g.) contained 1·27 g. glyceride. Correcting for losses and aliquots the total phospholipid-free fat (33·54 g.) contained 1·34 g. or 4·0% glyceride. On the basis of the glycerol actually isolated the glyceride content was only 44% of that estimated in the above manner. Therefore, 1·8% is the minimal and 4·0% is probably the maximal content of glyceride in the phospholipid-free fat.

Discussion

The results of this study of the acetone-soluble portion of the lipids confirm and extend the data published by Schoenheimer [1928]—the only other investigator to report upon the nature of the individual components of the lipid deposited in the tissues of atherosclerotic aortas. Schoenheimer examined material obtained by acetone-extraction of atherosclerotic aortas and utilized physical methods of separation (repeated fractional crystallization). In the present study only intimal tissue from atheromatous aortas was used; this was extracted with ether and the phospholipids were removed by acetone-precipitation. The methods of examination used (saponification followed by fractionation of the products or of derivatives) proved more suitable for the isolation of minor constituents than did the purely physical procedures used by Schoenheimer, and also permitted a nearly quantitative estimation of the amount of each.

In addition to stearic, palmitic and oleic acids (found by Schoenheimer as cholesteryl esters) we have isolated linoleic and arachidonic acids. No evidence of the presence of

linolenic acid was obtained. The amount of saturated acids was so small that we did not actually isolate pure stearic acid. However, the equivalents of the saturated acid fractions indicated that it was present.

A red viscous petroleum-insoluble acid mixture was also obtained which had I.V. 58·5. Attempts to isolate a pure substance from this were unsuccessful; its insolubility in petroleum suggested that it might be a hydroxy-acid. Several investigators have obtained similar fractions from the fatty acid mixtures of blood and blood plasma. Channon & Collinson [1929] removed this so-called resinous material before determining the constants of the fatty acids from ox blood, but they did not further describe its properties. From the fatty acids of beef, dog, sheep and pig plasma, Bloor [1923] removed acidic substances whose Pb salts were insoluble in hot alcohol. The free 'acid' had I.V. 24. Probably this material was of the same nature as that isolated from the lipids of atheromatous intimas.

Despite the fact that care was taken throughout the fractionation to protect the lipid from unnecessary exposure to air, it is quite possible that a certain amount of oxidation of the unsaturated acids could have taken place and given rise to petroleum-insoluble products. On the other hand the relative constancy of the yield of this fatty acid from individuals of the same species seems to indicate that its presence in the plasma fatty acids is characteristic and that it is not an artefact.

Of some interest is the question whether the highly unsaturated acids (arachidonic and linoleic) were derived from cholesteryl esters, glycerides or phospholipids. The lipid saponified (27·94 g.) actually yielded 175 mg. arachidonic acid and 783 mg. linoleic acid. These values did not represent the total amount of the two acids in the mixture, since it is well known that only a portion of each is precipitated in the α-form by bromination [Green & Hilditch, 1935]. The P content of the lipid saponified indicated that it contained only 0·39% phospholipid (calculated as lecithin) which could have yielded only about 76 mg. of total fatty acid. Therefore a large proportion (if not all) of the highly unsaturated fatty acids was derived from cholesteryl esters and/or glycerides. The latter, it has been shown, may possibly have contributed as much as 1·22 g. fatty acid. However, since glycerides seldom contain a single type of acid [Collin & Hilditch, 1929] it is unlikely that the highly unsaturated acids were derived solely from glycerides. The probability is that they were derived in part at least from cholesteryl esters.

The free acid, removed before saponification of the acetone-soluble lipid, had an equivalent weight of 491 and contained 3·6% P. While not enough of this material was available for fractionation, the above constants suggested that it might be largely phosphatidic acid (oleopalmitophosphatidic acid, mol. wt. 674·9, 4·5% P). This acidic fraction may have been either a precursor or a degradation product of the phospholipids.

A search of the literature failed to reveal a detailed report of a strictly comparable analysis of the lipid of human plasma for comparison with the data obtained from this study of atheromatous aortas. Csonka [1918], working with the total lipid of human plasma, found that the average I.V. of the 'liquid' acids was 87·5 and that this unsaturated fraction constituted about 48·5% of the total acid. Therefore the total acid had an I.V. of about 42. Even although this acid mixture included acids derived from phospholipids which are known to be more saturated than those in the acetone-soluble lipids of the plasma, the value seems to be low. Channon & Collinson [1929] obtained an I.V. of 120 for the fatty acid mixture derived from the acetone-soluble lipid of whole ox blood. The I.V. found here for the corresponding fatty acids of atherosclerotic intimal tissue was 104. On the assumption that the I.V. of Channon & Collinson's 'solid' acids was due to oleic acid and that the 'liquid' acids did not contain saturated acids, the total acid mixture was composed of 83% unsaturated and 17% saturated acids. This composition corresponds very closely with that of the fatty acid mixture from the acetone-soluble lipid of atherosclerotic intimal tissue (88% unsaturated and 12% saturated acids). Further-

more, the acetone-soluble lipids of ox blood and atheromatous intimal tissue yielded the same fatty acids, namely, stearic, palmitic, oleic, linoleic and arachidonic acids. In neither case was linolenic acid identified.

Such a comparison involving two different species may appear open to criticism. However, Bloor [1923] found a remarkable constancy in the ratio of 'solid' to 'liquid' acids in the fasting blood plasma of different species. He also found that the ratio of total fatty acid to unsaponifiable material of plasma lipid is relatively constant in different species, so that the comparisons made appear permissible.

Table 3 contains values (recalculated from several reports) for the lipids of atheromatous tissues, normal intimal tissue, human plasma, ox blood, human red blood cells and human white blood cells. The micro-analytical results of Boyd [1937], Page *et al.* [1935] and Weinhouse & Hirsch [1940] were recalculated to exclude phospholipids in order that they might be compared with the analytical results obtained in this study of

Table 3. *Percentage composition of the phospholipid-free lipids*

	Atheromatous intimas		Atheromatous aortas Schoenheimer [1928]		Ulcerated plaques Weinhouse & Hirsch [1940]	Early plaques Weinhouse & Hirsch [1940]	Normal intimas Weinhouse & Hirsch [1940]	Ox blood plasma (calculated) Channon & Collinson [1929]		Human plasma Page *et al.** [1935]	Human plasma Boyd [1937]	Human red blood corpuscles Boyd [1937]	Human white blood corpuscles Boyd [1937]
Total sterol	58·8		62·0		65·7	51·9	51·7	45·4		41·6	42·0	58·0	54·6
Bound sterol	34·5		35·7		31·4	30·4	31·9	29·3		26·3	29·0	3·7	13·4
Free sterol	24·3		26·3		34·3	21·5	19·8	16·1		14·7	13·0	54·3	41·2
Steryl ester	57·8		60·0		52·8	51·2	53·7	49·2		44·4	48·0	6·2	22·5
N.S.U.S.M.	4·6		3·7					8·3					
Free acid	5·5		—					—					
Glyceride	1·8–4·0	17·9	—	13·7	13·1	27·3	26·6	18·0	34·8	41·0	39·0	39·5	36·3
Unaccounted	3·8–6·0		10·0					8·5					
Free sterol/ bound sterol	0·70		0·74		1·09	0·71	0·62	(0·55) fixed		0·55	0·45	14·7	3·1

* Page, Kirk, Lewis, Thompson & Van Slyke.

the acetone-soluble lipids. Channon & Collinson's figures for whole ox blood were adjusted to exclude the free cholesterol from the red blood cells by fixing the ratio free sterol/bound sterol at a value of 0·55 (about the average value found for normal post-absorptive human plasma lipid), while the value given for bound sterol was kept relatively the same. This adjustment was made on the assumption that the red blood cells contained little or no steryl ester.

In the micro-procedures the fraction of the lipid mixture consisting of glycerides, non-sterol unsaponifiable material, free acid and lipid not accounted for was estimated only by difference. Therefore the relative amounts of its components are unknown. On the other hand the large scale analyses gave fairly reliable estimations of these components.

The table shows a close quantitative relationship between the values for the acetone-soluble lipid fractions of human blood plasma and those calculated for ox blood plasma, despite the difference in analytical technique and the assumptions made to render the comparison possible. Also, there is a remarkable similarity in the values for the sterol-containing fractions present in the lipids of atheromatous aortas and plasma, which confirms the observations of Weinhouse & Hirsch [1940] and Page [1941]. The most striking difference between the composition of the lipids from normal or atheromatous intimal tissue and that of the plasma is in the 'neutral' fraction. The acetone-soluble lipids from the intimal tissues contain 13–27% of 'neutral' lipid while the acetone-soluble lipids of the plasma, red blood cells and white blood cells contain 35–41% of this component. Since the non-sterol unsaponifiable material and free fatty acids are minor components of the acetone-soluble fraction, the difference in content of 'neutral' lipid in these two groups of lipids is due most likely to a difference in glyceride content. Direct estimation of the glyceride content of the lipids from atheromatous aortas (2–4%) and

ox blood (18%) gave data enchancing the validity of this supposition. Actually the mean differences are probably greater than is indicated by these figures since the plasma lipid was always obtained from blood in the fasting state, when the glyceride content is at a lower than average value. Therefore it would appear that the glycerides are either not absorbed from the plasma or they undergo subsequent decomposition in the intima.

Summary

1. The composition of the phospholipid-free fat from atheromatous intimal tissue of aortas has been determined.

2. The fatty acid mixture obtained by saponification of this lipid was found to be composed of 2·9% stearic acid, 14·6% palmitic acid, 65·2% oleic acid plus more highly unsaturated acids that escaped precipitation when brominated, 9·4% linoleic acid, 2·1% arachidonic acid and 5·8% petroleum-insoluble acids.

3. The glyceride content of this lipid, as evidenced by the amount of glycerol isolated and the amount of acid found in excess of that required for cholesteryl esters, was 1·8 and 4·0%.

4. A qualitative and quantitative comparison has been made between the phospholipid-free fat of atheromatous aortas and similar fractions from other sources in the body. The differences and similarities in these have been discussed.

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66. *l*(−)-Cysteic Acid Decarboxylase

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The pathway of taurine formation in the animal body is unknown. Friedmann [1903] showed that cystine is easily oxidized *in vitro* to cysteic acid and cysteic acid decarboxylated to taurine by the action of heat [see also White & Fishman, 1936], and he suggested the formation in the living body of taurine from cystine with cysteic acid as an intermediary. The increased output of taurocholic acid in dog's bile under suitable conditions after the administration of either cystine [Bergmann, 1904] or *l*(−)-cysteic acid [Virtue & Doster-Virtue, 1939] gives support to Friedmann's scheme, at least in the dog.

In experiments with tissue preparations from rat's liver an enzyme was found catalysing the oxidation of cystine and cysteine to cysteic acid [Bernheim & Bernheim, 1939; Medes, 1939], but attempts to demonstrate the presence of a decarboxylase for cysteic acid have been unsuccessful [Medes & Floyd, 1942]. In the following, evidence is given of the occurrence in mammalian liver of an enzyme which decarboxylates *l*(−)-cysteic acid.

Material and methods

The author is indebted to Dr A. Neuberger for the samples of *l*(−)-cysteic acid and *dl*-cysteic acid used in these experiments. The dog's livers were in most cases obtained from animals killed by the 'humane killer' and dissected soon after death. The cod's livers came from the Torry Research Station, Aberdeen. They were frozen as soon after death as possible and sent in a thermos flask to Cambridge where they arrived in a semi-frozen condition less than 48 hr. after the dispatch of the material. The human kidney extract was freshly prepared from material removed at operation [case C of paper by Blaschko, 1942]. The organs were weighed, ground in a mortar with sand, and equal amounts of *M*/15 phosphate buffer solution of *p*H 7·4 were added. The enzyme solution used was the supernatant fluid after 10–12 min. centrifuging.

Simple Barcroft-Warburg manometers were used; the flasks were conical with an inner tube and a side bulb. The main flask contained the extract, the side bulb the substrate (neutralized cysteic acid) and the inner tube *N* H_2SO_4. In each case a blank was set up with distilled water in the side bulb; the reading in this flask served for determining the CO_2 present in the extracts. All experiments were carried out at 37·5° and in N_2.

Experimental

When cysteic acid was added to dog's liver extracts there occurred an evolution of gas, starting at a uniform rate, but decreasing later and finally coming to a standstill. The total amount of gas evolved depended on the amount of cysteic acid added. The following observations indicate that the gas formed was CO_2: (1) when the readings had become constant and the extracts were acidified, a further amount of gas was set free from the vessel to which cysteic acid had been added, as compared with the control and, (2) when the inner tube was filled with alkali no development of gas occurred.

A typical experiment is shown in Table 1. The total CO_2 formed was one molecule per molecule of *l*(−)-cysteic acid added. This is the amount to be expected according to the equation:

$$\underset{\text{cysteic acid}}{HSO_3.CH_2.CH(NH_2).COOH} \longrightarrow \underset{\text{taurine}}{H.SO_3.CH_2.CH_2.NH_2} + CO_2.$$

Table 1. *Formation of CO_2 from l(−)-cysteic acid in dog's liver extract*

The contents of the side bulb were tipped into the main compartment at 0 min.; the contents were acidified at 50 min.

Flask no. ...	1	2
Main flask	1·8 ml. dog's liver extract	1·8 ml. dog's liver extract
Side bulb	0·2 ml. water	0·2 ml. *M*/100 *l*(−)-cysteic acid
Inner tube	0·3 ml. *N* H_2SO_4	0·3 ml. *N* H_2SO_4
μl. CO_2 0–50 min.	6·5	40·5
μl. CO_2 after acidification	13·5	28
μl. total CO_2	20	68·5
μl. CO_2 formed	—	48·5
μl. CO_2 expected	—	45

Distribution and activity. Dog's liver was chosen in the first instance because all observations on the excretion of taurocholic acid had been made on this animal. The choice proved convenient for other reasons. The extracts from dog's liver were most active and there was little CO_2 formation in the absence of substrate. The presence of a relatively large blank in the crude liver extracts of all other animals examined made the determination of the enzymic activity somewhat less accurate. This was especially marked in rat's liver where the activity of the enzyme was relatively high. The enzyme was found in the livers of the guinea-pig, the pig and the rat, but not in those of the cat, the rabbit and the cod. Extracts from the kidneys of the dog, cat and man were inactive.

The enzymic activity of the extracts was measured by determining the μl. CO_2 formed per mg. fresh tissue per hr. This is expressed as 'cysteic acid-$q^{N_2}_{CO_2}$' in Table 2. By multiplying the cysteic acid quotients by 5·58 the amounts of taurine formed can be calculated in mg. per g. fresh weight of tissue. These figures are also given in Table 2. They represent minimum values only, since some active material is lost in the preparation of the extracts.

Table 2. *l(−)-Cysteic acid decarboxylase activities of various liver extracts*

Animal	No. of experiments	Cysteic acid-$q^{N_2}_{CO_2}$	mg. taurine formed per hr. per g. tissue
Guinea-pig	2	0·045; 0·0525	0·25; 0·29
Pig	1	0·089	0·50
Rat	3	0·12 (0·073–0·180)	0·67 (0·41–1·00)
Dog	6	0·22 (0·11–0·33)	1·23 (0·61–1·84)

Stereospecificity. From *dl*-cysteic acid only half a molecule of CO_2 per molecule of substrate was formed. Since the total amount of CO_2 formed from the laevorotatory stereoisomeride was one molecule per molecule of substrate we may conclude that *d*(+)-cysteic acid was not attacked by the enzyme.

Inhibitors. The enzyme was inhibited by cyanide. With dog's liver extract the inhibition was complete in *M*/1000 HCN. The inhibition was of the reversible type. This was shown by the following procedure. Six manometers were used for the three parallel experiments of Table 3. The first two manometer flasks contained extract only, for the determination of its enzymic activity; to the other four flasks cyanide was added, but in flasks nos. 5 and 6 alkali and a filter paper were placed in the inner tubes in order to remove the HCN from the atmosphere over the extracts. Before the reaction was started the alkali and the filter paper were removed. Table 3 shows how the manometer flasks were set up. The flasks were attached to the manometers and filled with N_2; they were left standing at room temperature for 90 min. and then shaken in the thermostat at 37·5° for another 20 min. Filter paper and alkali were replaced by 0·3 ml. *N* H_2SO_4 in the inner tubes of flasks nos. 5 and 6. The rates of decarboxylation were then determined. The results are given in Table 3.

Table 3. *Inhibition by HCN* (M/*1000*)

Flask no. ...	1	2	3	4	5	6
Main flask:						
1·6 ml. dog's liver extract	+	+	+	+	+	+
0·2 ml. water	+	+	−	−	−	−
0·2 ml. *M*/50 HCN	−	−	+	+	+	+
Side bulb:						
0·2 ml. water	+	−	+	−	+	−
0·2 ml. *M*/50 *l*(−)-cysteic acid	−	+	−	+	−	+
Inner tube:						
0·3 ml. *N* H_2SO_4	+	+	+	+	−	−
0·3 ml. *N* KOH and filter paper	−	−	−	−	+	+
μl. CO_2 formed in 20 min.		48·5		0		43·5
Inhibition %		−		100 (with HCN)		10 (after HCN removed)

This experiment shows that during the incubation with alkali most of the HCN was removed from the enzyme with the result that the cyanide inhibition was almost completely reversed.

The enzyme was not inhibited by octyl alcohol.

Effect of dialysis. When dialysed against distilled water for 18 hr. at 0–2°, the extracts lost much of their activity. Addition of boiled extract did not increase the activity of the dialysed extracts.

Discussion

The experiments reported show that CO_2 is formed from *l*(−)-cysteic acid under anaerobic conditions. The fact that one molecule of CO_2 is formed per molecule of substrate can only mean that the reaction catalysed is the decarboxylation of *l*(−)-cysteic acid.

The presence of the enzyme in dog's liver would furnish a simple explanation of the observation that in this animal cysteic acid is excreted as taurocholic acid in the bile [Virtue & Doster-Virtue, 1939]. The enzyme has not been found in the livers of all animals which excrete taurocholic acid, e.g. the cat [Iwato & Watanabe, 1935] and the cod [Hammarsten, 1904]. Other pathways of taurine formation may exist [Medes & Floyd, 1942], and the possibility of formation of taurocholic acid without the intermediary appearance of free taurine has been discussed [Blum, 1904]. The failure of Medes & Floyd [1942] to find the enzyme in rat's liver is probably explained by the fact that they tested for decarboxylase activity in the insoluble residue only, thereby losing the active fraction in the supernatant fluid during repeated washings. Another difference was that their experiments were carried out in air and ours in N_2.

The decarboxylase for cysteic acid shows many similarities with dopa decarboxylase, an enzyme which occurs in the liver and kidney of many mammals and decarboxylates dihydroxyphenylalanine [Holtz, Heise & Lüdtke, 1938; Blaschko, 1939; 1942]. Both enzymes are reversibly inhibited by cyanide, they are not inhibited by octyl alcohol and they are stereospecific for the laevorotatory stereoisomeride. The two enzymes, however, must be different, as shown by their different distributions; cysteic acid decarboxylase does not occur in the human kidney. Their similarities indicate that the *l*-amino acid decarboxylases of vertebrates form a well-defined group with common properties.

Summary

Liver extracts of the dog, rat, pig and guinea-pig, but not of the cat, rabbit and cod, contain an enzyme, *l*(−)-cysteic acid decarboxylase, which forms CO_2 anaerobically from *l*(−)-cysteic acid, one molecule of CO_2 being formed from one molecule of *l*(−)-cysteic acid. The enzyme is stereospecific for the laevorotatory stereoisomeride, it is reversibly inhibited by cyanide, but insensitive to octyl alcohol. It is different from *l*(−)-dopa decarboxylase with which it shares common properties.

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67. Origin and Relationship of Acetylmethylcarbinol to 2:3-Butylene Glycol in Bacterial Fermentations*

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No general agreement exists as to the mechanism of the biological origin of acetylmethylcarbinol and 2:3-butylene glycol, although these products are formed by the dissimilation of various carbohydrates and polyhydric alcohols by many species of bacteria.

The purpose of the present communication is to contribute to our knowledge of the mechanism of the formation of acetylmethylcarbinol and 2:3-butylene glycol by *Aerobacillus polymyxa* (Prazmowski) Donker, an organism which produces considerable quantities of these products. Studies were made using as cultural media both glucose-peptone and glucose-peptone plus several probable intermediary compounds.

Desmots [1904] found acetylmethylcarbinol in cultures of *Bacillus mesentericus*. Organisms which produce acetylmethylcarbinol generally form 2:3-butylene glycol, the reduction product of the carbinol. Harden & Walpole [1906] isolated and described both 2:3-butylene glycol and acetylmethylcarbinol from the fermentation of glucose by *Aerobacter aerogenes*. Harden & Norris [1912] found that *Aerobacter aerogenes* and *Aerobacter cloacae*, when grown in a peptone solution containing any one of a series of sugars and polyhydric alcohols, produce both the carbinol and the glycol. Lemoigne [1919; 1923] found the carbinol to be formed by species of *Proteus* and by *Bacillus anthracis*. Neuberg & Reinfürth [1923], Kluyver, Donker & Visser't Hooft [1925] and Elion [1926] found it in yeast cultures. Lemoigne [1912], Lafon [1932], and Horowitz-Wlassowa & Rodionowa [1933] showed that the carbinol was present in cultures of *Bacillus subtilis*; van Niel, Kluyver & Derx [1929] detected it in cultures of *Streptococcus cremoris*, and first suggested the importance to flavour and aroma of the presence of acetylmethylcarbinol in butter cultures. Michaelian, Farmer & Hammer [1933] have conducted extensive investigations to show the role of diacetyl in butter aroma. Visser't Hooft & de Leeuw [1935] found the carbinol in bread which had a desirable aroma. These citations are but a few of the recorded examples.

Investigators are not agreed on the origin of acetylmethylcarbinol. Considerable evidence has been presented to support the view that acetaldehyde is an intermediary in the fermentation of glucose to acetylmethylcarbinol. Neuberg & Hirsch [1921], Neuberg & Ohle [1922], Neuberg & Reinfürth [1923], and Neuberg & Simon [1925] presented data showing that the carbinol is formed by the condensation of one molecule of added aldehyde with one molecule of biologically formed aldehyde. Neuberg attributes this condensation of aldehyde to the enzyme carboligase. In the normal yeast fermentation of glucose, no acetylmethylcarbinol or 2:3-butylene glycol is formed. Kluyver & Donker [1925] believe that acetaldehyde is a normal intermediate in this fermentation but that it is removed so rapidly that no condensation occurs. These investigators added other H acceptors, such as O_2, methylene blue and S, to compete with acetaldehyde, and in each case acetylmethylcarbinol and 2:3-butylene glycol were formed.

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Furthermore there is no general agreement as to whether at least one molecule of acetaldehyde must be nascent to form acetylmethylcarbinol. Elion [1926], starting with a 10% yeast suspension in 3% acetaldehyde, obtained a fair quantity of acetylmethylcarbinol in 3 hr. Barritt [1937] inoculated *Aerobacter aerogenes* and *Escherichia coli* into peptone water containing varying concentrations of acetaldehyde. Growth occurred in some of the media but acetylmethylcarbinol was not detected in any.

Formation of acetylmethylcarbinol from pyruvic acid by *Acetobacter* has been shown by Hermann & Neuschul [1932], and by *Clostridium acetobutylicum* by Johnson, Peterson & Fred [1933]. Pyruvic acid may be decarboxylated to form acetaldehyde, and the latter condense with pyruvic acid or with another molecule of itself to acetylmethylcarbinol.

Silverman & Werkman [1941], however, reported that a cell-free enzyme preparation from *Aerobacter aerogenes* converts pyruvic acid into acetylmethylcarbinol and CO_2 without detectable intermediate formation of acetaldehyde. It should be emphasized that the mechanisms of dissimilation with proliferating cells and with enzyme preparations may differ and that different species of bacteria may use different mechanisms of formation.

Other investigators differ as to the origin of acetylmethylcarbinol. Horowitz-Wlassowa & Rodionowa [1933] believe that with *Bacillus subtilis*, 2:3-butylene glycol results directly from splitting of the sugar molecule and that acetylmethylcarbinol is a product of biological oxidation of 2:3-butylene glycol. Lafon [1932] could detect neither pyruvic acid nor acetaldehyde in glucose cultures of *Bacillus subtilis* which produce acetylmethylcarbinol. He believed that the carbinol may originate from lactic acid or that, perhaps, there is a splitting of the hexose molecule to give the C_4 compound directly.

Several investigators have presented evidence that acetylmethylcarbinol originates from 2:3-butylene glycol by biological oxidation in the absence of a fermentable carbohydrate. Walpole [1910] was able to demonstrate the formation of the carbinol by cultivating *Aerobacter aerogenes* in a medium composed only of 2:3-butylene glycol, peptone and water. Lemoigne [1923] noted an increase of acetylmethylcarbinol, and a decrease of 2:3-butylene glycol in a *Proteus* culture after it was held for 18 days. Donker [1926] obtained the carbinol by growing *Aerobacillus polymyxa* aerobically in a medium composed only of yeast-water and 2:3-butylene glycol. Werkman [1930] inoculated a medium of 2:3-butylene glycol, $(NH_4)_2SO_4$, and K_2HPO_4 with several species of *Aerobacter*. Acetylmethylcarbinol was detected in cultures of all of the organisms on the second day. Barritt [1937] found that many bacteria can oxidize 2:3-butylene glycol to acetylmethylcarbinol and suggested that in the bacterial fermentation of glucose the glycol may precede the formation of the carbinol.

On the other hand, there is evidence that in the presence of fermentable sugar the reverse process takes place, namely, the reduction of acetylmethylcarbinol to 2:3-butylene glycol. Neuberg & Kobel [1925] added acetylmethylcarbinol to a fermenting mixture of sugar and yeast; the carbinol had disappeared by the third day and 2:3-butylene glycol was isolated from the fermented mixture. Nagelschmidt [1927] found acetylmethylcarbinol to be an intermediary product of the reduction of diacetyl to 2:3-butylene glycol by yeast. Visser't Hooft & de Leeuw [1935] found that under certain conditions the acetylmethylcarbinol in bread is reduced to 2:3-butylene glycol. According to Hammer, Stahly, Werkman & Michaelian [1935], under certain conditions the citric acid-fermenting streptococci of butter cultures reduce acetylmethylcarbinol to 2:3-butylene glycol.

Methods and materials

Acetylmethylcarbinol was determined by the method outlined by Stahly & Werkman [1936]. 2:3-Butylene glycol was determined according to Brockmann & Werkman [1933] and a correction made for the presence of any acetylmethylcarbinol [Stahly & Werkman, 1936]. Ethyl alcohol was determined according to Stahly, Osburn, & Werkman [1934]

with correction for acetylmethylcarbinol. The volatile acids were determined by the method of Osburn, Wood & Werkman [1933].

Culture. In all the experiments described in this paper, *Aerobacillus polymyxa* (Prazmowski) Donker [Donker, 1926] was used (culture no. 842 of the American Type Culture Collection).

Experimental

Effect of addition of acetaldehyde and acetate on yields of acetylmethylcarbinol and 2:3-butylene glycol

Freshly distilled aldehyde was added to a vigorously fermenting medium of 2% glucose, 1% $CaCO_3$, 0·5% peptone and 0·2% K_2HPO_4. The acetaldehyde, 0·708 g./300 ml. of medium, was added 40 hr. after inoculation. The controls contained basal medium without acetaldehyde. During the fermentation some of the media were aerated; others received a constant stream of O_2-free N_2. Some of the acetaldehyde may have escaped during the fermentation; none remained at the close.

The yields of 2:3-butylene glycol, acetylmethylcarbinol and ethyl alcohol (Table 1) were definitely greater in those cultures which received acetaldehyde. Aeration resulted in greater yields of acetylmethylcarbinol, both in the control and in the dissimilations receiving acetaldehyde, than occurred in strictly anaerobic experiments. Evidently added acetaldehyde is utilized by *Aerobacillus polymyxa* under the conditions of the experiment. Ethyl alcohol results, apparently, from the reduction of acetaldehyde.

Table 1. *Quantities of acetylmethylcarbinol, 2:3-butylene glycol and ethyl alcohol produced by* Aerobacillus polymyxa *in a glucose medium with and without added acetaldehyde*

Figures represent g. per 100 ml. of fermented liquor.

Condition	Medium	Glucose fermented	A.M.C.	2:3-B.G.	Total A.M.C. + 2:3-B.G.	Ethyl alcohol
Anaerobic	Control	1·96	0·017	0·483	0·501	0·342
,,	Control + acetaldehyde	1·99	0·050	0·580	0·631	0·472
,,	,,	1·98	0·034	0·529	0·563	0·491
Aerobic	Control	1·99	0·062	0·482	0·545	0·225
,,	Control + acetaldehyde	1·94	0·069	0·522	0·591	0·383
,,	,,	1·85	0·065	0·577	0·642	0·385

A.M.C. = acetylmethylcarbinol. 2:3-B.G. = 2:3-butylene glycol.

Additional evidence for the intermediary role of acetaldehyde was secured by the use of $CaSO_3$ as a fixing agent. A medium composed of 1% $CaCO_3$, 0·2% K_2HPO_4, 0·5% peptone, 2% glucose and 1% $CaSO_3$ was inoculated with *Aerobacillus polymyxa* and incubated at 30°. The fermented liquor was then distilled to one-half volume into a dilute ice-cold solution of dimedon. Crystallization of the derivative occurred slowly after several hours in the refrigerator. Melting point determinations indicated that the substance fixed was acetaldehyde.

An experiment was designed to determine whether the addition of acetate to a normal glucose fermentation by *Aerobacillus polymyxa* results in an increase of acetylmethylcarbinol and 2:3-butylene glycol. Both anaerobiosis and aeration were employed. The control medium was 2% glucose, 1% $CaCO_3$, 0·5% peptone and 0·2% K_2HPO_4. Calcium acetate, 2 g./300 ml., was added to the basal medium. Glucose was determined immediately after inoculation; and glucose, acetylmethylcarbinol and 2:3-butylene glycol after fermentation was complete. Table 2 gives typical experimental results.

It is apparent that added acetate results in a marked increase in the yield of 2:3-butylene glycol and acetylmethylcarbinol in the fermentation of glucose by *Aerobacillus*

Table 2. *Quantities of acetylmethylcarbinol and 2:3-butylene glycol produced by* Aerobacillus polymyxa *in a glucose medium with and without added acetate*

Figures represent g. per 100 ml. of fermented liquor.

Condition	Medium	Glucose fermented	A.M.C.	2:3-B.G.	A.M.C. + 2:3-B.G.
Anaerobic	Control	2·03	0·034	0·388	0·422
,,	Control + acetate	1·97	0·028	0·554	0·582
Aerobic	Control	1·97	0·036	0·466	0·502
,,	Control + acetate	1·98	0·071	0·626	0·697
,,	,,	1·99	0·073	0·630	0·703

polymyxa both aerobically and anaerobically. In every trial the total yield of acetylmethylcarbinol plus 2:3-butylene glycol was greater when calcium acetate was added. Aeration resulted in increased yields of acetylmethylcarbinol, both in the controls and in the medium receiving acetate. The results indicate that acetic acid plays a part in the biological formation of 2:3-butylene glycol and acetylmethylcarbinol. The intermediate mechanism is not evident; the acetic acid may first be reduced to acetaldehyde, and the latter may then condense with itself or pyruvic acid to acetylmethylcarbinol, which would be reduced to 2:3-butylene glycol.

The relationship of acetylmethylcarbinol to 2:3-butylene glycol in the glucose fermentation by Aerobacillus polymyxa

Experiments were designed to determine the fate of both acetylmethylcarbinol and 2:3-butylene glycol when they are added separately to glucose dissimilations by *Aerobacillus polymyxa*. Preliminary studies showed that vigorous oxygenation of a glucose culture of *Aerobacillus polymyxa* resulted in rapid fermentation, and that if acetylmethylcarbinol were added during the course of the fermentation, it was reduced largely to the corresponding glycol. In an attempt to secure a better understanding of the changes involved, it was decided to make serial determinations of glucose, acetylmethylcarbinol, 2:3-butylene glycol and E_h values during the course of typical fermentations. The medium was composed of 2% glucose, 1% $CaCO_3$, 0·3% peptone and 0·2% K_2HPO_4.

Three 2000 ml. Erlenmeyer flasks, each containing 1200 ml. of medium were fitted with five-hole rubber stoppers which held platinum electrode, KCl-agar bridge, gas outlet tube, gas inlet tube leading into an aerator device, and sampling tube. The apparatus was arranged to determine the redox potentials of the biological system according to the method of Werkman, Johnson & Coile [1933]. O_2 was continuously bubbled through the medium immediately after inoculation with *Aerobacillus polymyxa* until fermentation was completed. The cultures were incubated at 30°. Flask no. 1 served as the control; to flask no. 2 was added a definite quantity of sterile acetylmethylcarbinol 46 hr. after inoculation; and to flask no. 3 was added sterile 2:3-butylene glycol, 46 hr. after inoculation. The data are presented in Tables 3, 4 and 5.

The correlation of acetylmethylcarbinol and 2:3-butylene glycol with unfermented glucose and with E_h values is apparent from Fig. 1 *a* and *b*. The data are from Tables 3, 4 and 5.

Fig. 1 *b* represents the data obtained with flask 2. The results are typical of the two other fermentations. The data of this experiment show that in all three fermentations there was a rapid drop in redox potentials concomitant with a rapid decrease in the concentration of glucose. While the E_h value remained low, very little acetylmethylcarbinol accumulated, but there was a rapid rise in the quantity of 2:3-butylene glycol. In flask 1 (control) a marked increase in acetylmethylcarbinol and decrease in the rate of production of 2:3-butylene glycol occurred at about the 60th hr. The redox potential had become fairly high at that time, and the glucose was almost completely fermented.

The glycol content reached a maximum at the 70th hr. and then began to decrease. This decrease was almost exactly accounted for by the increase in acetylmethylcarbinol.

Table 3. *Quantities of acetylmethylcarbinol, 2:3-butylene glycol and glucose; and* E_h *values at intervals during the course of the fermentation of glucose by* Aerobacillus polymyxa

Flask 1—control.

Hours after inoculation	Unfermented glucose g./100 ml.	E_h, volts	Molarity A.M.C.	Molarity 2:3-B.G.	Molarity A.M.C. + 2:3-B.G.
0	1·99	0·113	0·0	0·0	0·0
24	1·19	−0·041	0·0005	0·0200	0·0205
32	—	−0·037	—	—	—
46	0·44	−0·047	0·0020	0·0461	0·0481
70	0·0	------	0·0102	0·0539	0·0641
80	0·0	+0·027	—	—	—
94	0·0	+0·200	0·0209	0·0429	0·0638
127	0·0	+0·177	—	—	—
142	0·0	+0·177	0·0244	0·0377	0·0621

Table 4. *Quantities of acetylmethylcarbinol, 2:3-butylene glycol and glucose; and* E_h *values at intervals during the course of the fermentation of glucose by* Aerobacillus polymyxa

Flask 2—A.M.C. added.

Hours after inoculation	Unfermented glucose g./100 ml.	E_h, volts	Molarity A.M.C.	Molarity 2:3-B.G.	Molarity A.M.C. + 2:3-B.G.
0	1·96	+0·247	0	0	0
8	—	+0·262	—	—	—
24	1·26	−0·233	0·0003	0·0198	0·0201
32	—	−0·266	—	—	—
46	0·30	−0·196	0·0020–0·0239*	0·0470	0·0490–0·0709
70	0·04	+0·093	0·0219	0·0646	0·0854
127	0·0	+0·307	—	—	—
142	0·0	+0·322	0·0325	0·0521	0·0846

* Sterile A.M.C. was added.

Table 5. *Quantities of acetylmethylcarbinol, 2:3-butylene glycol and glucose; and* E_h *values at intervals during the course of the fermentation of glucose by* Aerobacillus polymyxa

Flask 3—2:3-B.G. added.

Hours after inoculation	Unfermented glucose g./100 ml.	E_h, volts	Molarity A.M.C.	Molarity 2:3-B.G.	Molarity A.M.C. + 2:3-B.G.
0	1·87	+0·267	0	0	0
8	—	+0·327	—	—	—
24	1·26	−0·223	0·0008	0·0194	0·0202
32	—	−0·199	—	—	—
46	0·44	−0·173	0·0026	0·0465–0·0939*	0·0491–0·0965
70	0·0	—	0·0122	0·0947	0·1069
80	0·0	+0·089	—	—	—
94	0·0	+0·274	0·0263	0·0817	0·1080
127	0·0	+0·377	—	—	—
142	0·0	+0·382	0·0276	0·0793	0·1069

* Sterile 2:3-B.G. added.

The graphs for the fermentation in flask 2 show a definite decrease in the quantity of acetylmethylcarbinol after the carbinol was added and while the E_h value was still fairly low. This decrease was accounted for by the increase in glycol content in excess of that in flask 1 to which no carbinol had been added. To flask 3 was added 2:3-butylene glycol at the 46th hr. after inoculation. There was a slight rise after this in the glycol content but not as much as in flask 1. The rise in acetylmethylcarbinol occurred earlier and to a greater extent in flask 3 than in flask 1.

It is interesting to note that (1) passage of a stream of O_2 through these fermenting liquors did not prevent a rapid drop in E_h; (2) acetylmethylcarbinol added to the fermenting medium was quickly reduced to the corresponding glycol while the E_h remained low; (3) after most of the glucose had been fermented and the E_h had risen appreciably,

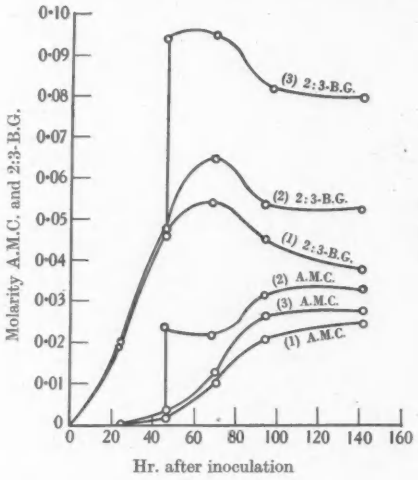

Fig. 1 *a*.

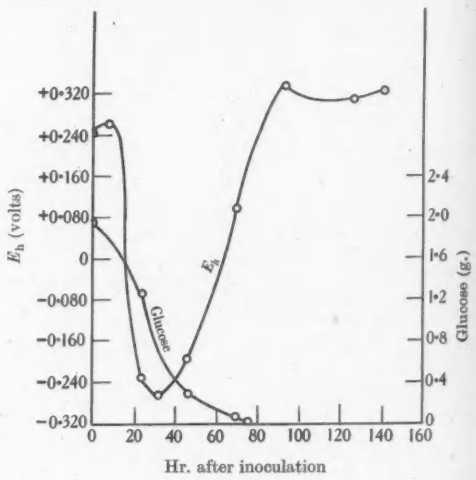

Fig. 1 *b*.

Fig. 1 *a*. Correlation of acetylmethylcarbinol and 2:3-butylene glycol with unfermented glucose and with E_h. Data for flasks nos. 1, 2 and 3.

Fig. 1 *b*. Correlation of acetylmethylcarbinol and 2:3-butylene glycol with unfermented glucose and with E_h. Data for flask 2.

much 2:3-butylene glycol was oxidized to the carbinol; and (4) after all the glucose had been fermented the total content of acetylmethylcarbinol plus 2:3-butylene glycol did not change significantly.

It is evident that a low redox potential favours the reduction of carbinol to glycol and a high potential favours the oxidation of the glycol to carbinol. It is near or at the time of the final disappearance of fermentable carbohydrate that conditions become suitable for the accumulation of acetylmethylcarbinol in the *Aerobacillus polymyxa* fermentation.

Summary and conclusions

1. In fermentations by *Aerobacillus polymyxa*, conducted either aerobically or anaerobically, the addition of acetaldehyde to a glucose-containing medium results in increased yields of ethyl alcohol, acetylmethylcarbinol and 2:3-butylene glycol.

2. Acetaldehyde is fixed by the addition of $CaSO_3$ to a normal fermentation. This evidence supports the probable intermediary role of the aldehyde.

3. Likewise, the addition of acetate results in increased yields of acetylmethylcarbinol and 2:3-butylene glycol.

4. A marked drop in redox potential and a rapid fermentation of the glucose follow inoculation of a vigorously oxygenated glucose medium with *Aerobacillus polymyxa*. The potential rises only after most of the glucose has been fermented.

5. Large quantities of 2:3-butylene glycol accumulate while the redox potential remains low; part of the glycol is oxidized to acetylmethylcarbinol after the E_h attains a fairly high level.

6. Little acetylmethylcarbinol accumulates during the early stages of the fermentation; added carbinol is reduced to glycol but only when the potential is low.

7. It is suggested that acetylmethylcarbinol and 2:3-butylene glycol comprise a reversible oxidation-reduction system. A low redox potential favours the accumulation of the glycol; a high potential favours carbinol formation.

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68. Studies on the Acetone-Butyl Alcohol Fermentation

2. Intermediates in the Fermentation of Glucose by *Cl. acetobutylicum*

3. Potassium as an Essential Factor in the Fermentation of Maize Meal by *Cl. acetobutylicum* (BY)

By **Ronald Davies**, *From the Biochemical Laboratory, Cambridge*

(*Received 3 July 1942*)

2. INTERMEDIATES IN THE FERMENTATION OF GLUCOSE BY *CL. ACETQBUTYLICUM*

The technique of preparing active cell suspensions of *Cl. acetobutylicum* (Weizmann) capable of giving normal fermentation products from glucose has been described [Davies & Stephenson, 1941]. It was stated that these suspensions rapidly lost their activity. In the present paper further experiments on the decay of activity are described and also various experiments on reactions performed by suspensions of the organism, in particular experiments relating to the reduction of butyric acid to butyl alcohol and to the formation of acetone.

Methods and technique

The analytical methods and the technique of growing the organism and preparing cell suspensions were the same as previously described [Davies & Stephenson, 1941] unless otherwise stated.

Estimation of acetone and acetoacetic acid in a mixture. To determine the acetone the sample was brought to *p*H 7·5 and the acetone distilled for 10 min. *in vacuo* into 25 ml. *N*/100 I_2+5 ml. *N* NaOH in the apparatus used for the estimation of acetone. Under these conditions acetoacetic acid does not distil. Acetone+acetoacetic acid was estimated by strongly acidifying the sample and distilling off the acetone by boiling at atmospheric pressure. The acetoacetic acid was obtained by difference.

The organism. Two strains of *Cl. acetobutylicum* have been used, the Weizmann strain and the BY strain; the particular strain used in a given experiment is indicated in each instance.

Experiments on the decay of activity of suspensions

Rate of decay. A suspension of cells was prepared and was kept in a test-tube in air at room temperature. After given intervals of time its activity was determined by measuring the rate of H_2 production from both glucose and pyruvate in Warburg manometers. The results are shown in Fig. 1 where the activity expressed as a percentage of the initial activity is plotted against time. The suspension had lost all activity towards glucose

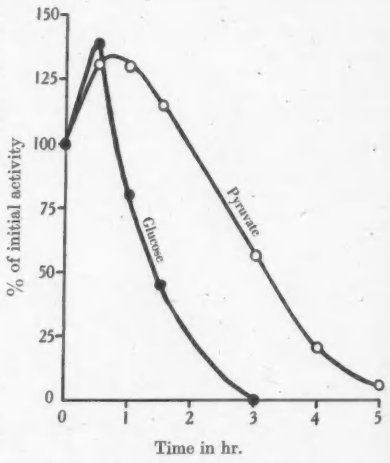


Fig. 1. Effect of time on activity of cell suspensions. *Organism:* Weizmann strain.

after 3 hr. and 96% of its activity towards pyruvate after 5 hr. The activity peaks shown in Fig. 1 were not observed in every experiment.

Table 1. *Effect of various substances and conditions on rate of decay*

In all cases activities were determined manometrically by measuring the Q_{H_2} over the first 5 min. Warburg manometers were used containing 0·6 ml. *M*/2 acetate buffer *p*H 5·0, 0·1 ml. cell suspension (20–30 mg. dry wt./ml. except in (*h*)), 1·8 ml. water and, in the side bulb, 0·3 ml. *M*/10 glucose. Gas phase H_2; temp. 37°.

In (*a*) and (*b*), 10 ml. cell suspension contained in each of four modified Thunberg tubes were gassed with the requisite gas for ½ min. and kept in an atmosphere of that gas; samples were expelled at intervals by forcing the corresponding gas into the tube.

In (*c*) and (*d*), 7 ml. cell suspension containing 1 mg./ml. of one of the substances named were kept in air in a test-tube at room temp.

In (*e*), (*f*) and (*g*), 10 ml. cell suspension containing glucose or galactose to the final concentrations given were kept in air in a test-tube at room temp. or at 0°.

In (*h*), 10 ml. cell suspension of the strengths given were kept in a test-tube in air at room temp.

The values of Q_{H_2} have been adjusted so that in each experiment the Q_{H_2} of the freshly prepared suspension was 100.

p-AB = *p*-aminobenzoic acid.

Organism: Weizmann strain.

Age of suspension hr.	(*a*) Air	O_2	N_2	H_2	(*b*) Air	H_2 + 5% CO_2	CO_2	(*c*) Air	Cysteine	Glutathione	*p*-AB
0	100	100	100	100	100	100	100	100	100	100	100
¾	16	0	31	32	47	50	73	100	97	81	89
1½	3	0	16	16	27	31	42	47	57	50	58
2¼	0	0	5	5	13	16	21	28	34	17	30
3	0	0	0	0	1	3	3	17	14	13	12

Age of suspension hr.	(*d*) Air	Nicotamide	Adenosine	Nicotinamide + Adenosine	Adenylic acid	(*e*) Air	*M*/100 Glucose	*M*/100 Galactose
0	100	100	100	100	100	100	100	100
¾	46	49	65	47	33	23	177	23
1½	22	25	35	30	20	6	104	6
2¼	0	3	11	14	5	0	66	0
3	0	0	0	3	0	0	39	0

Age of suspension hr.	(*f*) Air	*M*/100 Glucose	*M*/50 Glucose	*M*/25 Glucose	(*g*) Air 18°	Air 0°	*M*/100 Glucose 18°	*M*/100 Glucose 0°
0	100	100	100	100	100	100	100	100
¾	30	312	294	334	121	36	340	52
1½	6	320	394	448	48	14	346	23
2¼	4	142	310	440	19	4	194	15
3	0	86	222	441	8	0	4	11

Age of suspension hr.	(*h*) mg. dry wt. cells/ml. suspension 32·5	20·3	12·2	6·1	30·3	18·9	11·4	5·7
0	100	100	100	100	100	100	100	100
½	87	75	54	52	61	52	43	47
1	73	48	42	37	34	27	14	19
1½	53	—	—	33	20	23	23	22
2	—	—	—	—	11	12	12	12

Effect of anaerobiosis, temperature etc. on the rate of decay. The effect of storing the suspension in air, O_2, N_2, H_2, CO_2 and $H_2 + CO_2$ and the effect of storage temperature and of various compounds on the rate of decay has been studied. Experimental details and the results are given in Table 1. Mann & Quastel [1941] have shown that the destruction

of cozymase in brain tissue can be inhibited by the addition of nicotinamide to the tissue. Assuming that cozymase destruction might be responsible for the decay in activity of *Cl. acetobutylicum* suspensions the effect of nicotinamide was tried; it did not prevent decay, neither did adenosine nor the two together. Reducing agents such as cysteine and reduced glutathione were likewise ineffective. In fact the activity was maintained only when the cells were fermenting glucose. At 0°, where fermentation does not take place, even glucose was ineffective. The figures in Table 1 (*f*) show quite clearly that as the glucose was used up the protective action on the activity disappeared. At a concentration of *M*/100 the glucose had disappeared after 1½ hr., at *M*/50 after 2¼ hr. This was shown by the fact that there was no evolution of gas in the manometers during the second 5 min. of the equilibration period. At a concentration of *M*/25 the glucose had not disappeared after 3¾ hr.

Effect of cozymase. It was still considered possible that cozymase destruction was involved in the decay and to settle this point the cozymase in the cells was determined before and after a period of incubation at 37°, using the lactic dehydrogenase-diaphorase-methylene blue system. The results are given in Table 2. It is seen that cozymase was not

Table 2. *Stability of cozymase in cell suspensions*

Cell suspensions were prepared containing 0, 0·08 and 0·4 mg. added cozymase per ml. and 28·6 mg. cells per ml. The suspensions also contained *M*/50 acetate buffer *p*H 5·0. A portion of each suspension was immediately boiled and tested for cozymase in Thunberg tubes. The remainder of the suspension was incubated for 3 hr. at 37° and again tested for cozymase. The medium from which the cells had been harvested was passed through a Seitz filter and the filtrate also tested for cozymase.

The Thunberg tubes contained 1 ml. lactic dehydrogenase [Green, Needham & Dewan, 1937], 1 ml. diaphorase [Straub, 1939], 1 ml. *M*/15 phosphate buffer *p*H 7·5, 0·2 ml. *M* KCN, 0·1 ml. 0·5% methylene blue, 0·5 ml. cell suspension or other source of cozymase, and in the hollow stopper 0·1 ml. 2 *M* sodium lactate.

The fermentative activity of the cell suspension towards glucose was determined manometrically before and after incubation.

Organism: Weizmann strain.

	Initial reduction time	Reduction time after 3 hr. at 37°	Initial Q_{H_2}	Final Q_{H_2}
Cell suspension	10 min. 10 sec.	13 min. 10 sec.	112	0
Cell suspension + 0·08 mg. CoI/ml.	6 min. 50 sec.	9 min. 30 sec.	112	0
Cell suspension + 0·4 mg. CoI/ml.	3 min. 35 sec.	4 min. 35 sec.	112	0
Seitz filtrate	2 hr.	—	—	—
0·08 mg. CoI/ml.	7 min. 30 sec.	—	—	—
0·4 mg. CoI/ml.	2 min. 50 sec.	—	—	—
No cozymase	2 hr. 10 min.	—	—	—

destroyed to any appreciable extent during a period in which the glucose-fermenting activity fell to zero. The decay in activity therefore cannot be ascribed to destruction of cozymase; its cause remains to be found.

It is obviously impracticable in most experiments to use glucose as a stabilizing agent for the activity, and if reproducible results are to be obtained in the absence of glucose it is necessary so to arrange the experiment that the suspension is used within 15 min. of harvesting the cells.

Fermentation of possible intermediates

The action of cell suspensions on some substances that have been suggested as intermediates in the acetone-butanol fermentation has been investigated. 0·1 ml. portions of *M*/10 solutions of these substances were incubated with cell suspensions in Warburg manometers in an atmosphere of H_2 at *p*H 5·0 (and in the case of butyric acid *p*H 4·0) and the liberation of H_2 and CO_2 or the uptake of H_2 was measured. Both strains of organism were used. The substances examined were: glyceraldehyde, phosphoglyceric acid, hexosediphosphate, lactic acid, formic acid, methylglyoxal, acetoacetic acid, oxaloacetic

acid, fumaric acid, butyric acid, α-hydroxybutyric acid, β-hydroxybutyric acid, 2:3-dihydroxybutyric acid, glyoxylic acid, tetronic acid, crotonic acid, *trans*-γ-hydroxycrotonic acid, tetrolic acid and vinylacetic acid. Except for acetoacetic and oxaloacetic acids there was no evolution or uptake of gas in any instance although glucose and pyruvate were vigorously fermented. Therefore, with the two exceptions stated, these substances cannot be attacked by cell suspensions of *Cl. acetobutylicum* in the presence of H_2 only.

Reduction of unsaturated 4-C acids by cells actively fermenting glucose. The last four unsaturated acids in the list and fumaric acid were incubated with cell suspensions in the presence of glucose to see if they could be utilized when glucose was being fermented. 0·1 ml. portions of *M*/10 solutions of these compounds were incubated with 0·1 ml. *M*/10 glucose, 1·3 ml. *M*/5 acetate buffer *p*H 5·0 and 0·5 ml. cell suspension (30 mg. dry wt. cells/ml.) in an atmosphere of H_2 until fermentation of the glucose had ceased. The experiment was carried out in Warburg manometers and the H_2 evolved was measured and compared with that evolved from glucose alone. The residual unsaturated acid was then determined by measuring the H_2 taken up when a suitable aliquot was catalytically hydrogenated by means of palladium-charcoal. For this purpose Warburg manometers were used containing 0·5 ml. 5*N* H_2SO_4, 0·2 ml. palladium-charcoal suspension and 1·3 ml. water in the main compartment and 1·0 ml. fermentation fluid in the side bulb; the manometers were filled with H_2 (freed from traces of O_2 by passage over reduced copper heated in a furnace) and equilibrated for 15 min. at 37°. The contents of the side bulb were then mixed with the catalyst and the H_2 uptake measured. The presence of the cells slows down the hydrogenation somewhat but does not affect the amount of H_2 taken up and it was not found necessary to remove them [cf. W. A. Johnson, 1939]. Control hydrogenations on the cell suspension alone and on the completely fermented glucose were always carried out but were always negative.

The results (Table 3) show that with the possible exception of fumaric acid none of the compounds is utilized and therefore they do not function as intermediates in the fermentation of glucose.

Table 3. *Reduction of unsaturated 4-C acids in presence of glucose*

Substrate	H_2 from glucose and 4-C acid μl.	Initial 4-C acid μl.	Final 4-C acid μl.
Glucose	496	—	—
Glucose and vinylacetic acid	492	206	180
Glucose and crotonic acid	505	224	208
Glucose and *trans*-γ-hydroxycrotonic acid	500	224	222
Glucose and tetrolic acid	511	224	209
Glucose and fumaric acid	484	224	142

Organism: BY strain.

Oxaloacetic acid is rapidly attacked, the optimum *p*H for its decomposition being 4·0. The products of decomposition have not been determined but presumably it is first decarboxylated to pyruvic acid which is then broken down in the usual way.

The fact that formic acid is not attacked disposes of the theory that H_2 and CO_2 are produced from formic acid under the influence of formic hydrogenlyase. *Cl. acetobutylicum* is therefore similar to *Cl. tetanomorphum* and *Cl. welchii* in producing H_2 from glucose but not from formate [Woods & Clifton, 1937; Woods, unpublished].

Thunberg tube experiments using the methylene blue technique have shown that *Cl. acetobutylicum* possesses enzymes which can activate glucose, pyruvate and lactate as H_2 donators, but that the dehydrogenases for succinic and formic acids and for alcohol are absent (Table 4). An extremely active hydrogenase is present however, the most active suspension encountered having $Q_{\text{methylene blue; hydrogen}} = 12{,}000$.

Table 4. *Dehydrogenase activity of* Cl. acetobutylicum *suspensions*

Thunberg tubes contained 1 ml. cell suspension (4·2 mg./ml.), 1 ml. *M*/15 phosphate buffer *p*H 5·5, 1 ml. *M*/10 substrate, and in the stopper 0·5 ml. 0·1% methylene blue.

Substrate	$Q_{M.B.}$	Substrate	$Q_{M.B.}$
Glucose	106	Formate	0
Pyruvate	34	Succinate	0
Lactate	10	Ethyl alcohol	0

Organism: Weizmann strain.

Acetoacetic acid as an intermediate. Johnson, Peterson & Fred [1933] have shown that acetoacetic acid is rapidly broken down to acetone both in culture and by washed suspensions of *Cl. acetobutylicum*, the rate of attack being at a maximum when the rate of formation of acetone in the culture is also at a maximum. These findings have been confirmed by the manometric technique using Warburg manometers. 0·2 ml. *M*/10 acetoacetic acid was shaken with 0·2 ml. cell suspension (25 mg./ml.) in 2·6 ml. *M*/10 acetate buffer *p*H 5·0 in an atmosphere of H_2 and the CO_2 evolved was measured. One manometer contained 0·2 ml. 20% NaOH in the centre cup to show that the only gas evolved was CO_2. When CO_2 production had ceased the acetone and acetoacetic acid in the manometers were estimated as previously described; a control was done using boiled cells. The results are given in Table 5. It is clear that acetoacetic acid is enzymically decarboxylated quantitatively to acetone; the low yield of acetone may be accounted for by loss through vaporization while the manometers were shaking at 37°.

Table 5. *Decarboxylation of acetoacetic acid*

	Manometers		
	H_2 (boiled cells)	H_2	H_2 (NaOH in centre cup)
Acetoacetic acid decomposed, μl.	Nil	392	392
Acetone formed, μl.	Nil	372	351
CO_2 evolved, μl.	4	400	Nil

Organism: BY strain.

The optimum *p*H for acetoacetic acid decarboxylation was determined manometrically by measuring the rate of CO_2 evolution over the first 5 min. when cell suspensions were shaken with acetoacetic acid in acetate buffers. Fig. 2 shows that the optimum lies at about *p*H 4·3.

Fig. 3 shows the variation in acetoacetic decarboxylase activity of the cells with age of the culture. 2 l. of 2% glucose-liver medium were sown with 40 ml. of a 5% maize meal culture and incubated at 37° in an atmosphere of H_2. Samples were withdrawn at intervals for the preparation of cell suspensions. The decarboxylase activity of the suspensions was measured as described above. Up to 8 hr. no acetone could be detected in the culture by Rothera's test; from 9 to 12 hr. there was a slight reaction increasing in intensity; at 13 hr. there was 0·1 mg. acetone/ml. culture and by 27 hr. this had risen to 0·5 mg./ml. The activity of the cell suspension from the 27 hr. sample was very high ($Q_{CO_2} = 596$).

A few other properties of the acetoacetic acid decarboxylase are worth noting. (1) It will work aerobically just as well as anaerobically, thus differing from all the other enzymes of this organism so far studied. (2) It stands up to acetone treatment and in an acetone powder of whole cells retains its activity almost undiminished for months. (3) The enzyme can be extracted from the acetone powder by water at 37°.

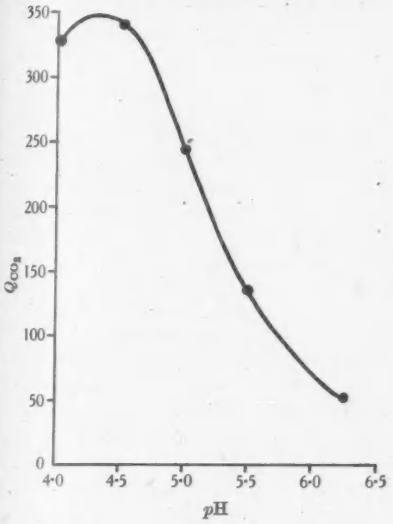


Fig. 2.

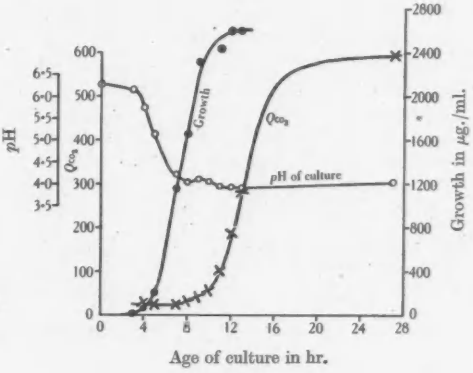


Fig. 3.

Fig. 2. Variation of acetoacetic acid decarboxylase activity with *p*H. The reactions were carried out in Warburg manometers containing 0·2 ml. cell suspension, 1·9 ml. water and 0·6 ml. *M*/2 acetate buffer of the desired *p*H in the main compartment; the side bulb contained 0·3 ml. *M*/10 acetoacetic acid (neutralized to *p*H 7·0). Gas phase air; temp. 37·5°. The *p*H measurements were made by means of the glass electrode. *Organism:* BY strain.

Fig. 3. Variation of acetoacetic acid decarboxylase activity with age of culture. The *p*H determinations were made by means of the glass electrode. Growth was estimated by means of a photoelectric turbidimeter. *Organism:* BY strain.

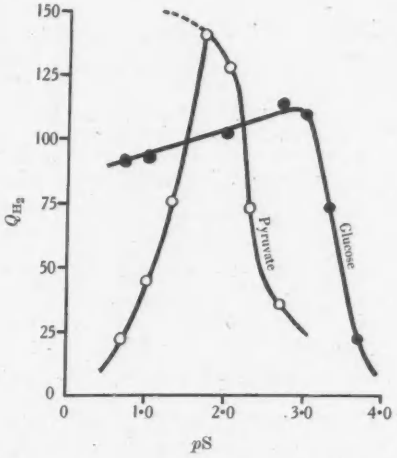


Fig. 4. Effect of substrate concentration on rate of fermentation. *Organism:* Weizmann strain.

Effect of substrate concentration on rate of fermentation

Glucose. The effect of substrate concentration on the rate of H_2 production from glucose was determined manometrically using Warburg manometers. Each manometer contained 0·6 ml. *M*/2 acetate buffer *p*H 5·0, 0·3 ml. cell suspension (10·8 mg./ml.); in the side bulb sufficient *M*, *M*/10 or *M*/100 glucose to give final concentrations ranging from *M*/5 down to *M*/5000. The centre cup contained 0·2 ml. 20% NaOH and the total volume of fluid was made up to 3·0 ml. with water; the manometers were filled with H_2 and shaken at 37°. The results are given in Fig. 4. The enzyme system displays a high affinity for its substrate, the concentration of glucose necessary to saturate the system being about *M*/1000; it may however be lower than this since, with final concentrations of glucose of *M*/2000 and *M*/5000, there is so little substrate present that the concentration may have been reduced below the saturation level before the zero reading could be taken after tipping the manometers.

Pyruvate. This was determined in the same way as for glucose, pyruvate at *p*H 5·0 being substituted for glucose. The affinity curve for pyruvate is included in Fig. 4. It is seen that concentrations of pyruvic acid greater than *M*/50 are inhibitory whilst at concentrations less than this the system is not saturated. The affinity of the pyruvate system for its substrate is seen to be much lower than that of the glucose system.

Fermentation of other sugars

In culture, *Cl. acetobutylicum* will ferment a number of carbohydrates and it was of interest to ascertain whether cell suspensions would ferment sugars other than glucose. The organism was therefore grown on the usual liver medium but with 2% maltose or 2% sucrose in the place of glucose. The ability of cell suspensions prepared from these cultures to ferment glucose, fructose, maltose and sucrose was then investigated by the usual-manometric technique. The results (Table 6) give a good example of adaptive and

Table 6. *Fermentation of sugars by* Cl. acetobutylicum

The manometers contained 0·6 ml. *M*/2 acetate *p*H 5·0, 0·3 ml. cell suspension (20 mg. dry wt./ml.), 2·0 ml. water, and in the side bulb 0·1 ml. *M* substrate. Gas phase H_2; temp. 37°.

	Substrate in manometer			
Substrate in growth medium	Glucose Q_{H_2}	Fructose Q_{H_2}	Maltose Q_{H_2}	Sucrose Q_{H_2}
2% glucose	450	143	21	0
2% maltose	454	—	298	37
2% sucrose	307	286	254	262

Organism: Weizmann strain.

constitutive enzymes. It is interesting that growing the cells on sucrose enables the cell to ferment maltose although the reverse is not the case. The adaptive nature of the sucrose-fermenting enzymes is of importance in connexion with the industrial fermentation of molasses.

Reduction of butyric acid to butyl alcohol

Without hazarding any theory on the production of 4-C compounds from glucose and pyruvate the problem of the reduction of butyric acid to butyl alcohol has been studied. It has already been shown manometrically that butyric acid is not reduced by H_2 in the presence of cell suspensions, and the fact that a considerable proportion of the glucose still remains to be fermented in a growth fermentation when the concentration of butyric acid starts to fall and butyl alcohol is rapidly being formed, suggested that the reducing agent might be glucose or some closely related substance. Experiments were therefore devised to show whether butyl alcohol could be formed from glucose and butyric and

acetic acids only, or whether some unknown intermediate formed in the early stages of the fermentation was essential to the reduction of butyric acid.

850 ml. of the usual medium containing 2% glucose were fermented until about half the glucose had disappeared. The culture was then divided into two parts with aseptic precautions, one half being allowed to ferment to completion. The other half was centrifuged so as to remove the cells and the medium was replaced by an equal quantity of fresh medium containing glucose, acetic and butyric acids in approximately the same concentrations as in the medium removed; the fermentation was then carried to completion, both cultures being analysed for the usual products. The result of one such experiment is given in Table 7. This shows that the cells from a half-fermented culture

Table 7. *Experiment on the reduction of butyric acid*

	Original culture initially	Same culture at half fermentation	New medium initially	New medium after complete fermentation	Original culture after complete fermentation
Conc. mg./ml. *p*H ...	6·29	4·03	4·03	4·09	4·24
Glucose	21·1	11·9	10·1	0·4	1·01
Acetone	None	0·15	None	0·79	1·22
Butyl alcohol	None	0·43	None	4·08	4·53
Ethyl alcohol	None	None	None	None	0·30
Butyric acid	None	3·04	3·36	1·55	0·69
Acetic acid	None	0·88	1·05	1·59	1·24

Organism: Weizmann strain.

when incubated with only the glucose, butyric and acetic acid constituents of the culture produce butyl alcohol in about the normal amount. Therefore it can be said that the reduction of butyric acid is not dependent on any unknown intermediate produced during the first half of the fermentation.

Glucose as the reducing agent. The ability of glucose to function as the reducing agent of butyric acid was then examined using cell suspensions. The reaction envisaged was:

$$C_6H_{12}O_6 + C_3H_7.COOH = 2CH_3.CO.COOH + C_4H_9.OH + H_2O. \quad (1)$$

The pyruvic acid formed would then be fermented chiefly to acetic acid (see Table 9) according to the equation:

$$CH_3.CO.COOH + H_2O = CH_3.COOH + CO_2 + H_2, \quad (2)$$

the over-all reaction being:

$$C_6H_{12}O_6 + C_3H_7.COOH + H_2O = 2CH_3.COOH + 2CO_2 + 2H_2 + C_4H_9.OH. \quad (3)$$

The experiment was made as set out in Table 8. The vessel contents were analysed for acetone, butyl alcohol, acetic and butyric acids and residual glucose. It is seen from Table 8 that (1) no reduction of butyric acid by H_2 alone occurs; (2) in the presence of glucose and butyrate the amount of butyl alcohol formed is greater than that produced

Table 8. *Reduction of butyric acid by cell suspensions in the presence of glucose*

The reactions were carried out in Krebs vessels, each containing 10 ml. cell suspension (14·5 mg. dry wt./ml.) and in addition (*b*) contained 8 ml. *M*/2 butyric acid adjusted to *p*H 5·0 with NaOH, (*c*) 4 ml. *M* glucose, and (*d*) 4 ml. *M* glucose and 8 ml. *M*/2 butyrate *p*H 5·0. The total volume was made up to 40 ml. with distilled water in each case. All vessels were gassed with H_2 and incubated for 18 hr. at 38°.

Conc. mg./ml.	(*a*) No substrate	(*b*) Butyrate	(*c*) Glucose	(*d*) Glucose + butyrate
Initial glucose	None	None	18·5	18·5
Initial butyric acid	None	8·8	None	8·8
Glucose fermented	None	None	16·3	10·93
Butyl alcohol	0·36	0·28	3·61	4·18
Acetic acid	0·09	None	0·88	3·72
Butyric acid	0·13	8·65	0·83	5·13

Organism: Weizmann strain.

by the fermentation of glucose alone. The glucose fermented in this experiment (10·93 mg.) would, by itself, have produced 2·18 mg. butyl alcohol; 3·82 mg. were formed whilst 3·52 mg. butyric acid disappeared. The ratio (mol. glucose fermented/mol. butyl alcohol formed) is 2·06 for glucose alone and 1·18 for glucose + butyrate; equation (3) predicts a ratio of 1·0. The relationship between glucose and butyric acid disappearing and butyl alcohol and acetic acid formed is not quantitative, this would hardly be expected in such a complex system, but the evidence seems fairly good that glucose or some substance closely related to it is the reducing agent for butyric acid. Results which parallel these have been reported by Osburn, Brown & Werkman [1938] for the fermentation of glucose in the presence of butyric acid by growing cultures of *Cl. butylicum*.

The same relationships between glucose and butyric acid disappearing and butyl alcohol and acetic acid formed would hold if the active reducing agent was pyruvic acid and not glucose, assuming that glucose first breaks down to pyruvic acid. The equation for the reduction would be:

$$C_3H_7.COOH + 2CH_3.COCOOH + H_2O = 2CH_3.COOH + 2CO_2 + C_4H_9.OH. \quad (4)$$

In this connexion it may be noted that Brown, Osburn & Werkman [1937] have shown that suspensions of *Cl. butylicum* can bring about the reduction of butyric acid in the presence of pyruvate at *p*H 4·8 but not at *p*H 6·6. *A priori* evidence against this mechanism for *Cl. acetobutylicum* fermentations is afforded by the nature of the reaction products when (*a*) glucose, (*b*) pyruvate are fermented by *ripe* cell suspensions. Pyruvic acid gives rise chiefly to acetic acid with some acetone and butyric acid but scarcely any butyl alcohol (Table 9). To decide whether pyruvic acid could function as the reducing agent for butyric acid an experiment was carried out in which glucose and pyruvate were fermented (*a*) alone, (*b*) in the presence of butyric acid. The results are given in Table 10.

Table 9. *Products of fermentation of glucose and pyruvate by suspensions of ripe cells*

Product	Glucose mol./mol. glucose fermented	Pyruvate mol./2 mol. pyruvate fermented	Product	Glucose mol./mol. glucose fermented	Pyruvate mol./2 mol. pyruvate fermented
CO_2	2·46	2·077	Acetone	0·141	0·077
H_2	1·87	1·87	Acetic acid	0·124	1·53
Butyl alcohol	0·615	0·011	Butyric acid	0·037	0·106
Ethyl alcohol	0·006	0·049			

The reactions were carried out in Krebs vessels containing 10 ml. cell suspension, 10 ml. *M*/5 phosphate buffer *p*H 5·0, 6 ml. *M* glucose or 24 ml. *M*/2 pyruvate (adjusted to *p*H 5·0) and water to a total volume of 60 ml. Gas phase H_2; temp. 38°. Incubated for 18 hr. and then analysed. CO_2 and H_2 were measured in Warburg manometers using 1/20 the above quantities.

Organism: BY strain.

Table 10. *Glucose and pyruvate as reducing agents for butyric acid*

The reactions were carried out in Krebs vessels containing 10 ml. cell suspension (16·5 mg. dry wt./ml.), 2 ml. *M*/2 butyric acid (adjusted to *p*H 5·0) in (*b*) and (*d*), 1 ml. *M* glucose in (*c*) and (*d*), 2 ml. *M* pyruvic acid (adjusted to *p*H 5·0) in (*a*) and (*b*). The volume in each case was made up to 40 ml. with water. Gas phase H_2; temp. 38°. Incubated for 18 hr.

The products of fermentation are expressed as mol./mol. glucose fermented or mol./2 mol. pyruvate fermented.

Product	(*a*) Pyruvate	(*b*) Pyruvate + butyrate	(*c*) Glucose	(*d*) Glucose + butyrate	Product	(*a*) Pyruvate	(*b*) Pyruvate + butyrate	(*c*) Glucose	(*d*) Glucose + butyrate
Acetone	0·094	0·117	0·143	0·129	Butyric acid	0·233	+0·135*	0·161	−0·333†
Butyl alcohol	0·048	0·046	0·513	0·749	Acetic acid	1·67	1·57	0·392	0·645

* 0·135 mol. butyric acid have been formed per mol. pyruvate fermented.
† 0·333 mol. butyric acid have disappeared per mol. glucose fermented.

Organism: Weizmann strain.

It is seen that butyric acid is not reduced by pyruvate; in fact it appears to have no appreciable effect on the course of pyruvate fermentation. With glucose the results are similar to those already given in Table 8. On the basis of these results it may be suggested as a working hypothesis that triosephosphate functions as the reducing agent for butyric acid in the same way as it does for acetaldehyde in alcoholic fermentation by yeast. Attempts to isolate phosphoglyceric acid by fermenting glucose in the presence of butyrate and NaF according to the method of Stone & Werkman [1937] have so far been unsuccessful; possibly the correct conditions have not yet been found. Neither has it been possible to ferment hexosediphosphate with or without adenylic acid as phosphate acceptor, nor to bring about the reduction of butyric acid by hexosediphosphate. These last results are, however, of no great significance as hexosediphosphate does not easily penetrate the cell wall and is, for example, not readily attacked by intact yeast cells.

Intermediates in acetone production

Early experiments have shown that acetone production by the growing organism is increased by an autolysate of liver or maize [Davies & Stephenson, 1941]. In an attempt to concentrate the active substance precipitation by basic lead acetate was used and the filtrate, after removal of the excess Pb by CO_2, gave with glucose a much increased acetone production when incubated with cell suspensions in the usual way. This was finally attributed to the acetic acid which remained after removal of the excess Pb (Table 11). The effect of acetic acid on acetone production from pyruvate was then

Table 11. *Effect of acetic acid on acetone production*

The reactions were carried out in Krebs vessels each containing 1 ml. *M* glucose, 7 ml. cell suspension (12 mg. dry wt./ml.), 4·2 ml. 2 *M* acetate buffer *p*H 5·0 in (*b*), 4·2 ml. liver preparation 2* in (*c*), 4·2 ml. liver preparation 2 and 4·2 ml. 2 *M* acetate in (*d*), 4·2 ml. lead-free filtrate as described in the text in (*e*), and 4·2 ml. tryptic digest of liver containing the equivalent of 1 mg. fresh liver per ml. and 4·2 ml. 2 *M* acetate in (*f*). The total volume in each vessel was made up to 21 ml. with water. Gas phase H_2; temp. 38°. Incubated for 18 hr.

Addenda	Glucose fermented mg./ml.	Acetone formed mg./ml.	Acetone as % glucose fermented
(*a*) No factor; no acetate	9·0	0·33	3·7
(*b*) No factor; acetic acid 24 mg./ml.	9·16	1·57	17·2
(*c*) 20% liver preparation 2; no acetate	9·88	0·34	3·4
(*d*) 20% liver preparation 2; acetic acid 24 mg./ml.	9·85	1·74	17·7
(*e*) 20% lead-free filtrate; no acetate	10·16	1·63	16·1
(*f*) 20% tryptic digest of liver and acetic acid 24 mg./ml.	10·00	1·92	19·2

* *Liver preparation* 2. The lead-free filtrate described in the text was acidified to *p*H 2 with H_2SO_4 and the volatile acids removed by three distillations with water *in vacuo*; the H_2SO_4 was removed quantitatively with $Ba(OH)_2$ and the fluid concentrated *in vacuo* so that 1 ml. = 1 g. liver.

Organism: Weizmann strain.

examined together with the corresponding effect of butyrate; blanks with acetate and butyrate alone were also carried out. The results (Fig. 5) show that here too the presence of acetate resulted in a large increase in acetone formation from pyruvate; butyrate was ineffective and there was no formation of acetone from acetic or butyric acids either alone or together. With similar concentrations of acetic acid (24 mg./ml.) the increase in acetone in both cases was about 4–5 fold. Pyruvic acid can often be demonstrated by the Simon test in cultures grown on glucose and it is probably an early intermediate in glucose fermentation [cf. Brown *et al.* 1937]. Attention was therefore directed to the further examination of the reaction between pyruvate and acetate.

The effect of acetate concentration on acetone production from pyruvate by cell suspensions is shown in Fig. 6 together with the corresponding curves for glucose. It is

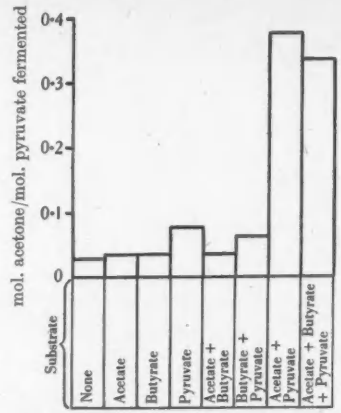


Fig. 5. Effect of acetate and butyrate on acetone production from pyruvate. The reactions were carried out in Krebs vessels containing 7 ml. cell suspension (20 mg./ml.), 0·7 ml. 0·7 *M* pyruvic acid, and where required 4·2 ml. 2 *M* acetate buffer *p*H 5·0 and/or 4·2 ml. *M*/2 butyric acid *p*H 5·0; the total volume was made up to 21 ml. with water. Gas phase H_2; temp. 37°. Incubated for 18 hr. and then analysed for acetone. *Organism:* Weizmann strain.

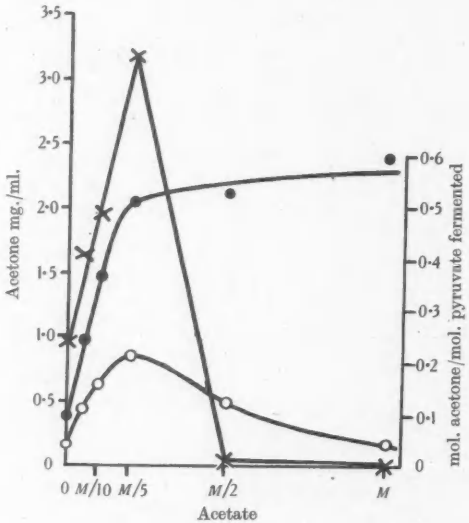


Fig. 6.

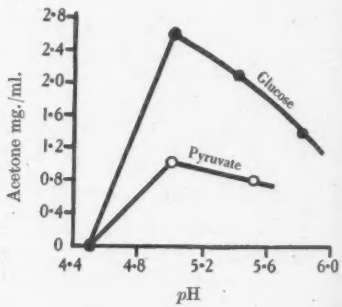


Fig. 7.

Fig. 6. Effect of acetate concentration on acetone production. The reactions were carried out in Krebs vessels containing 2 ml. *M* glucose or 1 ml. 0·6 *M* pyruvic acid, 5 ml. cell suspension (15 mg. dry wt./ml.), and sufficient 2 *M* acetate buffer *p*H 5·0 to give final acetate concentrations of nil, *M*/20, *M*/10, *M*/5, *M*/2, and *M*; the total volume was made up to 20 ml. with water. Gas phase H_2; temp. 38°. Incubated for 18 hr. and then analysed for acetone. *Organism:* Weizmann strain. o—o Pyruvate as substrate (acetone mg./ml). •—• Pyruvate as substrate (acetone mols./mol. pyruvate fermented). ×—× Glucose as substrate (acetone mg./ml.).

Fig. 7. Effect of *p*H on acetone production in the presence of *M*/5 acetate. The conditions were the same as under Fig. 6 except that all vessels contained 2 ml. 2 *M* acetate buffer of the desired *p*H. *Organism:* Weizmann strain.

seen that the maximum formation of acetone from both substrates occurs with a final acetate concentration of $M/5$ and that the ratio mol. acetone/mol. pyruvate fermented attains a maximum steady value of about 0·5–0·6. The optimum *p*H for acetone formation in the presence of $M/5$ acetate was then determined by incubating cell suspensions with glucose or pyruvate as substrate in the presence of $M/5$ acetate buffer of the required *p*H. Fig. 7 shows that the optimum production of acetone occurs at *p*H 5·0.

Role of acetic acid in acetone production. There are a number of references in the literature to the fact that the addition of acetic acid to a maize-meal or a glucose fermentation by *Cl. acetobutylicum* gives rise to increased amounts of acetone [Reilly, Hickinbottom, Henley & Thaysen, 1920; Speakman, 1920] and the hypothesis that acetone is formed from acetic acid via acetoacetic acid has been accepted by most workers [Reilly *et al.* 1920; Bernhauer & Kurschner, 1935; Osburn *et al.* 1938; Donker, 1926; van der Lek, 1930]. It has been shown that acetoacetic acid is almost certainly the immediate precursor of acetone. If the acetoacetic acid arises from condensation of two molecules of acetic acid, as previous workers have suggested, then the condensation reaction must be one of two coupled reactions for, as is seen from Fig. 5, acetic acid alone gives rise to no appreciable amounts of acetone when incubated with cell suspensions. If pyruvic acid or glucose is present as well, then large amounts of acetone are formed. The concentration of acetic acid necessary to produce these large amounts of acetone is very high and the relationship between acetate concentration and acetone production rather suggested that the acetic acid was functioning, not by taking part in a coupled reaction, but rather by exerting a mass action effect, suppressing a particular line of breakdown of glucose or pyruvic acid and/or increasing the reaction which gives rise to acetoacetic acid. This idea agrees well with a theory of acetone formation based on the scheme of Krebs & Johnson [1937] and indicated in the following equations [see Kalckar, 1941]:

$$CH_3.CO.COOH + CH_3.COOH = CH_3.CO.CH_2.CO.COOH + H_2O, \quad (5)$$

$$CH_3.CO.CH_2.CO.COOH + H_2O = CH_3.CO.CH_2.COOH + CO_2 + H_2. \quad (6)$$

Acetopyruvic acid as an intermediate in acetone formation. Acetopyruvic acid was prepared from sodium ethyl acetopyruvate [Claisen & Stylos, 1887] by the method of Mumm & Bergell [1912], and its ability to function as an intermediate in the formation of acetone was examined by incubating a solution with a cell suspension and measuring gas production and acetone formation. Details are given in Table 12. From this experi-

Table 12. *Attempt to ferment acetopyruvic acid*

Warburg manometer vessels contained 1 ml. $M/5$ phthalate-phosphate* buffer *p*H 5·0 or 0·3 ml. $2M$ acetate buffer *p*H 5·0, 1 ml. cell suspension in the main compartment; 0·1 ml. M acetopyruvic acid (adjusted to *p*H 5·0) and/or 0·1 ml. $0{\cdot}64M$ pyruvic acid (*p*H 4·5) in the side bulb. The total volume was made up to 3·0 ml. with water. Gas phase H_2; temp. 37°.

The acetone experiments were carried out in Krebs vessels containing 7 ml. cell suspension, 7 ml. phthalate-phosphate buffer and 0·7 ml. $0{\cdot}64M$ pyruvic acid in (*c*), 7 ml. phthalate-phosphate buffer and 0·7 ml. M acetopyruvic acid in (*a*) and (*b*), 2·1 ml. $2M$ acetate buffer and 0·7 ml. M acetopyruvic acid in (*d*), 2·1 ml. $2M$ acetate buffer and 0·7 ml. $0{\cdot}64M$ pyruvic acid in (*e*). The total volume was made up to 21 ml. with water in each case. Gas phase H_2; temp. 38°.

Acetopyruvic acid at *p*H 5·0 did not interfere with the acetone estimations.

Organism: Weizmann strain.

Substrate	Q_{gas}	Acetone mg./ml.
(*a*) Acetopyruvic acid (boiled cells)	0	0·038
(*b*) Acetopyruvic acid (phthalate-phosphate buffer)	0	0·057
(*c*) Pyruvic acid (phthalate-phosphate buffer)	156	0·117
(*d*) Acetopyruvic acid (acetate buffer)	0	0·084
(*e*) Pyruvic acid (acetate buffer)	183	0·597
(*f*) Acetopyruvic acid and pyruvic acid (phthalate-phosphate buffer)	61	Not done

* $M/5$ phthalate buffer containing 10% $M/5$ KH_2PO_4, *p*H finally adjusted to 5·0.

ment it is seen that (1) acetopyruvic acid is not attacked under the conditions used; (2) acetopyruvic acid inhibits the fermentation of pyruvic acid by about 60% at the concentration used ($M/30$ acetopyruvic and $M/50$ pyruvic). There still remained the possibility that acetopyruvic acid was toxic in concentrations as high as $M/30$; the manometric experiments were therefore repeated using $M/300$ and $M/3000$ acetopyruvic acid but again there was no fermentation. These experiments seem to show that acetopyruvic acid is not an intermediate in the formation of acetone.

In order to find out whether acetopyruvic acid could be fermented when added to fermenting cultures the following experiments were made. 400 ml. 2% glucose in the usual medium were inoculated with 10 ml. of a 5% maize culture and incubated for 20 hr. by which time fermentation was proceeding vigorously; 8 ml. of 10% acetopyruvic acid adjusted to *p*H 5·0 were then added and the incubation continued. The culture was analysed for glucose and acetone before the addition of the acetopyruvic acid and again at the end of the incubation. It was found that the addition of the acetopyruvic acid inhibited the further fermentation of the glucose and itself gave rise to no acetone (Table 13). A similar experiment was done on 5% maize mash and gave a similar result.

Table 13. *Effect of acetopyruvic acid on the fermentation of glucose by a growing culture of* Cl. acetobutylicum

The acetopyruvic acid was added to the culture immediately after the removal of the 20 hr. sample.

	Initial	20 hr.	110 hr.		Initial	20 hr.	110 hr.
*p*H	6·70	4·03	3·89	Acetone mg./ml.	—	Trace	0·064
Glucose mg./ml.	22·0	17·3	16·0				

Organism: Weizmann strain.

When the same amount of acetopyruvic acid was added to the medium before inoculation, growth could not be initiated. It is stated that compounds containing the grouping —CO—CH_2—CO— are unable to pass through the cell wall [Krampitz & Werkman, 1941]; acetopyruvic acid is such a compound and this might serve to explain why it appears not to function as a precursor of acetone were it not for the fact that it inhibits growth and fermentation. This seems to be incompatible with the theory that the acid cannot diffuse through the cell wall.

From these experiments it seems unlikely that acetopyruvic acid is an intermediate in the fermentation; some phosphorylated form of the substance may however be involved. The inhibitory effects may be due to competitive inhibition of processes in which pyruvic acid itself is concerned.

The possibility of acetone formation from acetic acid through direct condensation to acetoacetic acid was now further examined. Because no acetone is formed from acetic acid alone, whereas it is formed in increased amounts when acetic acid or glucose is also present, it must be assumed that the condensation of acetic acid to give acetoacetic acid requires energy which can be supplied by glucose or by pyruvate. On this theory the increased acetone formation from glucose or pyruvate and acetate should be accompanied by a disappearance of acetate. Accordingly, fermentations of glucose and of pyruvate in the presence of $M/5$ acetate *p*H 5·0 by cell suspensions were carried out and full analyses made of the products of fermentation. The results (Table 14) show that there has been a utilization of acetic acid in both cases, but the utilization is greater with glucose and acetate than with pyruvate and acetate. With glucose and acetate there has also been a considerable increase in the formation of butyric acid.

The figures in Table 14 show that about 0·5 mol. of acetone is formed for every mol. pyruvic acid fermented in the presence of $M/5$ acetate at *p*H 5·0. It follows that if the acetone has arisen from acetoacetic acid produced according to equation (7)

$$2CH_3.COOH = CH_3.CO.CH_2.COOH + H_2O, \qquad (7)$$

Table 14. *Effect of M/5 acetate on products of fermentation of glucose and pyruvate*

The reactions were carried out in Krebs vessels containing 15 ml. cell suspension, 6 ml. 2 *M* acetate buffer *p*H 5·0, 6 ml. *M* glucose, 11 ml. *M* pyruvate (adjusted to *p*H 5·0). The total volume was made up to 60 ml. with water. Gas phase H_2; temp. 38°. Incubated for 18 hr. Experiments were also done with no substrate and with acetate only as substrate; the figures given below have been corrected for these blanks.

The products are expressed as mol./mol. substrate fermented.

	Substrate					Substrate			
Product	Pyruvate	Pyruvate and acetate	Glucose	Glucose and acetate	Product	Pyruvate	Pyruvate and acetate	Glucose	Glucose and acetate
Acetic acid	0·68	−0·066*	0·206	−0·771*	Butyl alcohol	0	0	0·547	0·540
Butyric acid	0·115	0·054	0·082	0·280	Ethyl alcohol	0	0	0	0·137
Acetone	0·111	0·473	0·125	0·491					

* 0·066 and 0·771 mol. of acetic acid have been used up per mol. pyruvate and glucose fermented respectively.

Organism: Weizmann strain.

then the whole of the pyruvic acid disappearing must have been broken down to acetic acid, since the total concentration of acetate is almost unchanged. This agrees with the idea that the breakdown of pyruvic acid to acetic acid supplies the energy for the condensation of acetic acid to acetoacetic acid; it does not explain why such a high concentration of acetic acid is required to bring the acetone production up to the level of 1 mol. per 2 mol. pyruvate fermented. It seems that the mechanism of acetone formation still remains to be solved, but the solution must involve pyruvic and acetic acids together.

Acetoacetic acid as an intermediate in butyric acid formation

The association of an increased production of butyric acid with disappearance of acetic acid when glucose is fermented in the presence of *M*/5 acetate suggested that acetic acid was also involved in butyric acid formation and in particular that butyric acid might be

Table 15. *Acetoacetic acid and butyric acid formation*

The reactions were carried out in Krebs vessels containing 12 ml. *M*/5 phosphate buffer *p*H 5·65, 10 ml. cell suspension (2·95 mg. dry wt./ml.), 2 ml. *M*/5 glucose in (*a*), 4 ml. *M*/10 acetoacetic acid in (*b*) and 2 ml. *M*/5 glucose + 4 ml. *M*/10 acetoacetic acid in (*c*). The total volume was made up to 30 ml. with water. Gas phase N_2; temp. 37°. Incubated for 2½ hr. A control was also done with no substrate and the figures given in the table have been corrected for this.

CO_2 and H_2 production were measured in Warburg manometers using 1/20 the above quantities.

	Substrate		
Product mg.	Glucose (*a*)	Acetoacetic acid (*b*)	Acetoacetic acid and glucose (*c*)
Glucose fermented	68·0	—	68·0
H_2	1·49	Nil	1·69
CO_2	29·9	2·36	36·5
Acetic acid	10·42	Nil	10·42
Butyric acid	20·68	Nil	20·68
Acetone	Nil	Not done	8·96 (3443 μl.)
Acetoacetic acid disappearing	—	Not done	16·76 (3681 μl.)
CO_2 excess in (*c*) over (*a*)	—	—	3360 μl.

Organism: BY strain.

formed by the reduction of acetoacetic acid. This idea was tested in the following way. A cell suspension was prepared using the cells from a young culture (6 hr.); these cells are capable of decarboxylating acetoacetic acid at only a comparatively slow rate ($Q_{CO_2} = 16$), but are able to form butyric acid very rapidly from glucose; they cannot form butyl alcohol or acetone. The cells were therefore in such a condition as greatly to

favour the reduction of acetoacetic acid to butyric acid if this reaction was possible. This cell suspension was then incubated in Krebs vessels with (*a*) glucose, (*b*) acetoacetic acid, (*c*) glucose + acetoacetic acid, until fermentation ceased. The reaction mixtures were analysed for acetic and butyric acids, acetone, acetoacetic acid and residual substrate. H_2 and CO_2 production was measured in Warburg manometers using 1/20th the amounts of substrates in the Krebs vessels. The results (Table 15) show that acetoacetic acid has not been reduced, the whole of that disappearing being recovered as acetone.

3. POTASSIUM AS AN ESSENTIAL FACTOR IN THE FERMENTATION OF MAIZE MEAL BY *CL. ACETOBUTYLICUM* (BY)

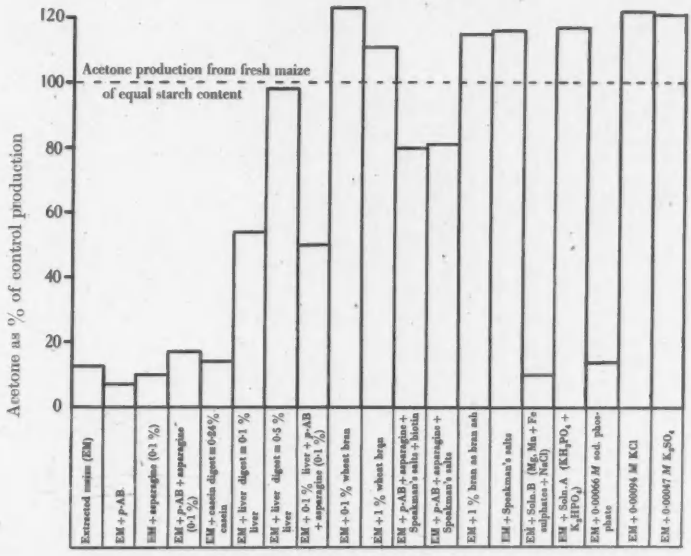
It has been reported [Weizmann & Davies, 1937] that if maize meal is extracted by shaking at room temperature with 0·2% HCl or *N*/10 $NaHCO_3$, fermentation of the resulting maize is very much slowed down although the yields of solvents are not markedly affected. The addition of fresh maize or of yeast autolysate restores the fermentation to normal.

Experimental

Finely ground maize meal was shaken mechanically for 2 hr. with 8 times its weight of freshly prepared *N*/10 $NaHCO_3$. The larger particles were allowed to settle, the supernatant fluid was carefully decanted and centrifuged for 10 min. at 3500 r.p.m. The deposit from the centrifuge was put back with the rest of the maize and the whole extraction process repeated twice more. After the third extraction the maize was suspended in about 1 l. distilled water and any residual $NaHCO_3$ neutralized with *N* HCl until the *p*H was about 6·0. The maize was then filtered off on a Büchner funnel, filtration being rapid and the filtrate water-clear, and dried at about 70° for several hours. Finally it was finely ground in a small coffee grinder. Quantities of 4 g. of this extracted maize, together with any additional substances, were made up into a mash with distilled water and diluted to 80 ml.; the media were contained in long tubes 325 × 28 mm. Each tube was sown with 1 ml. of a 24 hr. culture of *Cl. acetobutylicum* (BY) on 5% maize mash. Incubation at 37° was continued until fermentation had ceased (90–100 hr.) and the cultures were then analysed for acetone and residual starch. The weight of starch fermented was obtained approximately by the following method. Aliquots of the vigorously shaken culture and of the extracted and the fresh maize were hydrolysed in 10 volumes *N* HCl at 100° for 1 hr. and the liberated glucose was determined by Hanes's method [1929]. The difference between the glucose figures before and after fermentation gives the glucose equivalent of the starch fermented. The method is quick and sufficiently accurate for the purpose. In all experiments fermentations with extracted maize were compared with a fermentation of fresh maize carried out at the same time. Usually only the yield of acetone was taken as the measure of the normality of the fermentation, but in several instances butyl alcohol was also estimated and was found to confirm the acetone figures. The results (Fig. 8) have been expressed as 100 × (acetone produced from extracted maize)/(acetone produced from fresh maize of equal starch content).

The poor fermentability of $NaHCO_3$-extracted maize was confirmed. The addition of *p*-aminobenzoic acid was then tried and it was found to be without effect on either the degree of fermentation or the yield of acetone. This finding was repeated five times with four different preparations of extracted maize and *p*-aminobenzoic acid concentrations from 1 to 30 μg./ml. Asparagine (0·1%), with or without *p*-aminobenzoic acid, was likewise ineffective. The extraction of the maize removes a considerable proportion of its protein and the effect of adding sufficient tryptic digest of casein to replace this was tried;

this too was ineffective. A tryptic digest of liver was found to be very active, the yield of acetone being restored to normal by the addition of 0·5 % of liver. *p*-Aminobenzoic acid with or without asparagine did not increase the effect of suboptimal amounts of liver. Wheat bran was also very efficient, 1 % being even more effective than 1 % liver in that with bran the fermentation was just as rapid as that of fresh maize, whereas with liver it was definitely slower although the final acetone yields were normal in both cases (see Table 17). The effect of biotin methyl ester was next examined, this substance being the only known essential growth factor for this organism not yet tried. In case a multiplicity of factors was involved the biotin was tested at a level of 0·0004 μg./ml. in the presence of asparagine (0·1 %), *p*-aminobenzoic acid (1 μg./ml.) and Speakman's salts. A control


Acetone as % of control production
120
100
80
60
40
20
0
Acetone production from fresh maize of equal starch content
Extracted maize (EM)
EM + p-AB
EM + asparagine (0·1 %)
EM + p-AB + asparagine (0·1 %)
EM + casein digest = 0·5 % casein
EM + liver digest = 0·1 % liver
EM + liver digest = 0·5 % liver
EM + 0·1 % liver + p-AB + asparagine (0·1 %)
EM + 0·1 % wheat bran
EM + 1 % wheat bran
EM + p-AB + asparagine + Speakman's salts + biotin
EM + p-AB + asparagine + Speakman's salts
EM + 1 % bran ash
EM + Speakman's salts
EM + Sols. B (Mg, Mn + Fe sulphates + NaCl)
EM + Soln. A (KH_2PO_4 + K_2HPO_4)
EM + 0·0066 M sol. phosphate
EM + 0·0094 M KCl
EM + 0·0047 M K_2SO_4

p-AB = *p*-aminobenzoic acid, 1 μg./ml.

Fig. 8. Effect of various substances on the fermentation of $NaHCO_3$-extracted maize. For the composition of Speakman's salt mixture, see McDaniel, Woolley & Peterson [1939]. Solution B consisted of the Mg, Mn and Fe sulphates and the NaCl constituents of Speakman's salts. Solution A consisted of the K_2HPO_4 and the KH_2PO_4 constituents of Speakman's salts, giving a final [K] in the medium of 0·0094 *M* and a final $[PO_4]$ of 0·0066 *M*. *Organism:* BY strain.

was done omitting the biotin. Surprisingly, the control showed a much improved fermentation and the addition of biotin gave no further improvement. Since asparagine and *p*-aminobenzoic acid together had already been shown to be without effect it was concluded that (*a*) the improved fermentation was due to Speakman's salts, (*b*) biotin was not involved. Confirmation that the failure of extracted maize to ferment is due to a lack of certain salts was obtained by adding to the extracted maize ashed bran equivalent to 1 % bran, when fermentation was restored to normal in so far as the yield of acetone was concerned. It was still somewhat slower than that of fresh maize. The components of Speakman's salt mixture were next tested and the whole effect was found to reside in the potassium phosphate fraction and specifically in the potassium ion, sodium phosphate being completely inactive and both KCl and K_2SO_4 fully active. Finally, K estimations were made on samples of fresh and extracted maize and the results are shown in Table 16. Extraction of the maize had reduced the K content to less than 1/100th of the amount in

Table 16. *Analysis of fresh and extracted maize*

Aliquots of maize were dry-ashed in a muffle furnace and the residue taken up in 2 *N* HCl. Potassium was estimated in the resulting solution by the method of Kramer & Tisdall [1921].

	K content of maize %	Concentration of K in a 5% mash *M*		K content of maize %	Concentration of K in a 5% mash *M*
Fresh maize	1·113	0·0143	Extracted maize	0·0082	0·0001

fresh maize and the concentration in a 5% mash of extracted maize to 0·0001 *M*. When this concentration was increased to 0·001 *M* by the addition of KCl or K_2SO_4 the maize was fermented with a normal yield of acetone.

Visual observation showed that initially some fermentation of the extracted maize does take place and it appears to be just as rapid as that of fresh maize, but it soon comes to a standstill. This suggests that the residual K in the maize permits of some fermentation and that the cells take up K during growth. When all the available K has been exhausted growth and fermentation cease. Although the final yield of acetone can be restored to normal by K salts the speed of fermentation of extracted maize in the presence of K salts is still appreciably slower than that of fresh maize; 1% bran will restore both the speed and the solvent yield (Table 17). This suggests that besides K there is yet another factor

Table 17. *Rate of fermentation of extracted maize*

	Analyses			
	40 hr.		112 hr.	
Culture	Acetone mg.	Starch fermented g.	Acetone mg.	Starch fermented g.
1. Fresh maize	204	2·46	225	2·66
2. Extracted maize (E.M.)	3	0·23	17	0·35
3. E.M. and 1% liver digest	90	1·30	209	2·36
4. E.M. and 0·5% wheat bran	153	1·61	200	2·79
5. E.M. and 1% wheat bran	240	2·49	261	2·99
6. E.M. and Speakman's salts	100	1·45	236	2·67
7. E.M. and Speakman's salts, *p*-AB, asparagine and biotin	87	0·95	110	1·51
8. E.M. and 0·0066 *M* K phosphate	93	1·29	278	2·63
9. E.M. and 0·0094 *M* KCl	98	1·34	280	2·76

Organism: BY strain.

which is present in suboptimal amounts in extracted maize. This has not been further investigated but it is evidently not asparagine, *p*-aminobenzoic acid, or biotin, or any combination of these.

Summary of Parts 2 and 3

1. Factors affecting the rate of decay of activity of suspensions of *Cl. acetobutylicum* have been investigated; the cells maintain their activity only so long as they are fermenting glucose.

2. The fermentation of maltose and sucrose by cell suspensions has been shown to be dependent on the presence of these sugars in the growth medium. The cells are capable of fermenting glucose irrespective of whether they are grown on glucose, maltose or sucrose.

3. Glyceraldehyde, phosphoglyceric acid, hexosediphosphate, lactic acid, formic acid, methylglyoxal, acetoacetic acid, oxaloacetic acid, fumaric acid, butyric acid, α-hydroxybutyric acid, β-hydroxybutyric acid, 2:3-dihydroxybutyric acid, glyoxylic acid, tetronic acid, crotonic acid, *trans*-γ-hydroxycrotonic acid, tetrolic acid and vinylacetic acid have

been examined as possible intermediates in the fermentation of glucose. Of these only acetoacetic acid and oxaloacetic acid are attacked with the production of CO_2 and/or H_2.

4. None of the above compounds can be reduced by H_2 in the presence of cell suspensions able to ferment glucose.

5. Acetoacetic acid is rapidly decarboxylated to acetone by suspensions of ripe cells; with young cells the decarboxylation is slow.

6. Vinylacetic, tetrolic, *trans*-γ-hydroxycrotonic, crotonic and fumaric acids are not appreciably attacked by cells which are actively fermenting glucose. It is therefore concluded that they are not intermediates in the fermentation of glucose.

7. Acetoacetic acid is not reduced by cells which are actively fermenting glucose under conditions where its decarboxylation is slow; therefore it is probably not the precursor of butyric acid.

8. Butyric acid is reduced to butyl alcohol by cell suspensions in the presence of glucose but not in the presence of pyruvate. It is concluded that glucose or some closely related substance, possibly triosephosphate, is the reducing agent for butyric acid.

9. The formation of acetone from glucose and from pyruvate by cell suspensions is increased 4–5 fold by the presence of $M/5$ acetate at pH 5·0; acetic acid alone does not give rise to acetone.

10. The increased acetone is accompanied by a utilization of acetic acid in both cases but this is greater with glucose than with pyruvate.

11. Acetopyruvic acid is not attacked by cell suspensions and is inhibitory to the fermentation of glucose, maize meal and pyruvate.

12. Potassium has been shown to be an essential factor in the fermentation of maize meal by *Cl. acetobutylicum*. It is probably necessary for the growth of the organism.

I wish to express my sincere thanks to Dr M. Stephenson for a great deal of valuable advice and encouragement throughout the course of this work; to Prof. Friedmann for his expert guidance in the preparation of acetopyruvic acid; to Dr E. Watchorn for her kindness in carrying out the potassium estimations on maize meal; to Dr H. A. Krebs and Dr A. Kleinzeller for very kindly supplying me with a number of the substances examined in the course of this work; to Dr C. Weizmann for his co-operation and for his kindness in supplying me with the BY strain of *Cl. acetobutylicum*. I also wish to express my gratitude to the Medical Research Council for a full time grant.

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69. The Effect of the *p*H of the Medium during Growth on the Enzymic Activities of Bacteria (*Escherichia coli* and *Micrococcus lysodeikticus*) and the Biological Significance of the Changes Produced

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Researches of recent years on the enzymic activities of micro-organisms have shown that the bacterial cell possesses a very variable enzymic constitution. Karström [1938] pointed out that bacterial enzymes can be divided into two main classes: adaptive enzymes which are formed by the cell in response to the presence of the specific substrate, and constitutive enzymes which are formed whether the corresponding substrate is present during growth or not. This covers only one aspect of enzyme variation as both adaptive and constitutive enzymes undergo considerable variation with the physical conditions holding during growth. Thus the activities of the cells may alter with the temperature and degree of anaerobiosis during growth, the age of the culture and the presence in the medium of substances which are not related to the enzyme substrate, etc. The present communication deals with the alterations in enzyme activities of two organisms, *E. coli* and *M. lysodeikticus*, with the *p*H of the medium in which they are grown.

Gale [1940*a*; 1941] has shown that certain bacteria are able to produce amines by the decarboxylation of amino-acids by specific decarboxylases. These enzymes, studied in suspensions of washed intact organisms, have *p*H optima at unusually low values lying between 2·5 and 5·5, and are only formed by the organisms when growth occurs in acid media, the lower the *p*H the greater the resulting decarboxylase activity within physiological limits. Silverman & Werkman [1941] have shown that *A. aerogenes* can decompose pyruvic acid in two ways: a hydroclastic breakdown to acetic and formic acids and a second mechanism involving the condensation of two pyruvic acid molecules to acetylmethylcarbinol. The carbinol system has an optimal activity in the vicinity of *p*H 5 and is formed only in response to an acid growth environment, so that if the organism is grown in a medium maintained at an alkaline reaction, then the formation of the carbinol system is completely suppressed. The results are due to a true variation in the enzyme content of the cells as they are confirmed by experiments carried out with enzyme preparations obtained by disintegration of the cells with powdered pyrex glass. Earlier observations by Kocholaty & Hoogerheide [1938] on certain dehydrogenation reactions obtained with *Cl. sporogenes* suggested that the optimum activity *p*H of certain dehydrogenases, e.g. alanine and pyruvic dehydrogenases, can shift with alteration of the growth *p*H. A similar result is claimed by Kocholaty & Weil [1938] for the proteases of *Cl. histolyticum* where the *p*H optimum of the excreted protease is stated to shift from 7 when growth occurs in casein broth, to 6 when growth occurs in the presence of glucose. It is not possible to tell from the results in this case whether more than one protease is involved and, as it appears that glucose can alter the enzymic constitution of bacteria apart from any *p*H effect involved in its fermentation [Epps & Gale, 1942], these results

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require further elucidation. In the organisms studied, we have been unable to show any significant shift of the optimum *p*H of any enzyme with alteration of the growth *p*H, and it seems that this factor is a characteristic of the enzyme rather than of the culture in which it is produced.

Methods

The results described in this paper were obtained with the 'original *Escherichia coli*' National Collection of Type Cultures no. 86, and with *Micrococcus lysodeikticus* no. 2665. The basal medium used throughout was tryptic digest of casein with the addition of 0·5% NaCl and, in the case of *M. lysodeikticus*, 1% marmite. For the greater part of the work, growth took place in flasks incubated at 27° but *M. lysodeikticus* is difficult to grow in liquid media and in order to get sufficient material this organism was grown in Roux bottles containing 70 ml. medium, lying on their sides and incubated at 37°. *E. coli* will grow under these conditions at any *p*H between 4·5 and 9 although the yield of organism towards the limits of this range is small and it was sometimes impossible to harvest cultures grown at *p*H 4·5. The media were adjusted to the desired *p*H before growth by the use of a glass electrode and the *p*H was again checked when growth had finished. The metabolic activities of the organisms tend to alter the *p*H of the medium so that there is a drift towards neutrality from either side. This drift may amount to as much as 1·5 *p*H units at the end of the range and for the purpose of this work it was necessary to prevent the drift as far as possible. The best way to accomplish this was found to be the incorporation of buffer in the medium and the following final concentrations of buffer were used: for media adjusted to *p*H 4·5 and 5, *M*/60 phthalate; for *p*H 6–8, *M*/45 phosphate, and for *p*H 8·5 and 9, *M*/60 borate. These additions do not interfere with the growth of the organisms and we have satisfied ourselves that the results to be presented are not due to, or affected by, the presence of the buffers. This point has been checked in all possible cases by repetition of the estimations as far as possible with cultures grown in media adjusted without buffers and by checking the activity of the *p*H 7 culture with that of a culture grown at *p*H 7 in the presence of *M*/60 phthalate and borate; in no case has there been any significant effect due to the presence of buffers as such during growth. Even with the addition of these amounts of buffer to the media, there is still a small drift in *p*H during growth and the extent of this drift is shown for a typical example in Table 1. The organisms were harvested at the end of growth, washed

Table 1. *Variation of* p*H of media during growth of* E. coli

Buffer	Initial *p*H	Final *p*H	Mean *p*H	Buffer	Initial *p*H	Final *p*H	Mean *p*H
M/60 phthalate	4·55	4·71	4·63	*M*/45 phosphate	8·21	7·82	8·01
M/60 phthalate	4·95	5·58	5·26	*M*/60 borate	8·82	8·70	8·75
M/45 phosphate	6·16	6·58	6·37	*M*/60 borate	9·10	9·10	9·10
M/45 phosphate	7·01	7·10	7·05				

in water and made up into washed suspensions in water. The dry weight of the suspensions was determined by the use of a photoelectric turbidimeter previously calibrated against the organisms concerned.

The methods used for the estimation of enzymic activity differ with the system concerned and so will be described in the main text. It is desirable in work of this nature to restrict the investigation to single enzymes so that complication of the results does not arise owing to different types of variation of parts of an enzyme system, and so it is necessary to choose the estimation technique in each case so that the results obtained may be ascribed with reasonable certainty to the action of a single enzyme.

Range of p*H of medium in which* E. coli *will grow*

Fig. 1 shows the variation of the generation time of *E. coli* with the *p*H of the medium during growth. The generation time was estimated by the use of the photoelectric turbidimeter, the amount of organism present being estimated every 20 min. from the first appearance of turbidity to the end of the logarithmic phase of growth, and thus represents the time taken for the dry weight of organism to double itself within the logarithmic phase. This method involves certain errors due to variation in size of the

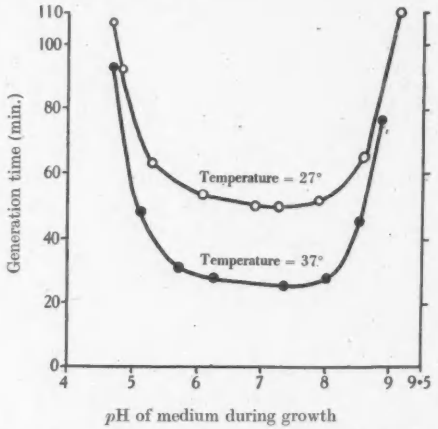

Fig. 1. Variation of generation time with growth *p*H (*E. coli*).

individual organisms in the early stage of growth but is sufficiently accurate for the purpose required. *E. coli* is able to grow at any *p*H between 4·5 and 9, the generation time outside these limits being so long that growth is insignificant. The generation time is unaffected by growth *p*H as long as the latter falls between the approximate limits 5·8–8·0. Fig. 1 also shows the effect of growth temperature on the generation time at all growth *p*H values.

Maintenance of cultures

At first it was thought that it might be possible to adapt the organism to various growth *p*H values. Accordingly cultures were grown at various values of *p*H within the growth range; each was subcultivated daily for one month into media of the same *p*H, and then used for inoculation of the medium at the same *p*H used for bulk experiments. The activities of such cultures were compared with those inoculated from neutral media, with results shown in Table 2. It can be seen that cultures inoculated from organisms 'trained' at *p*H 5·3 and 8·9 and grown at those *p*H values have substantially the same activities as cultures grown at *p*H 5·3 and 8·9 but inoculated from organisms maintained

Table 2. *Effect of maintenance* p*H of culture on activities*

*p*H of medium ...	5·3	8·9	5·3	8·9
Inoculum grown at *p*H ...	7·0	7·0	5·0	9·0
Enzyme:				
Arginine decarboxylase	264	0	238	0
Ornithine decarboxylase	374	0	320	0
Lysine decarboxylase	233	0	242	0
Histidine decarboxylase	22	0	16	0
Glutamic acid deaminase	5	17	5	14
Formic dehydrogenase	138	319	147	272

in neutral media. Likewise we have found that it is not possible to extend the growth *p*H range by 'training' at the extreme values. Accordingly this method of maintaining the cultures was abandoned and media were inoculated from cultures in normal neutral medium.

To avoid complications due to age of culture effects, it has been our custom to inoculate the media at the ends of the growth range a few hours earlier than the remainder and all cultures have been harvested as nearly as possible at the end of the period of active cell division, when activities are maximum for the culture. In order that activities determined for cultures grown at various *p*H values might be comparable, it has also been the custom to deal with one enzyme at a time and to grow cultures over the full *p*H range simultaneously. To avoid dilution effects, suspensions prepared from each day's set of cultures have been adjusted to approximately the same dry weight of organism per ml.

The results set out below for *E. coli* have all been obtained with suspensions of intact organism and consequently all activities are referred to the enzymes concerned as estimated in the intact organism and *p*H values refer to those holding in the external environment.

Enzymes concerned in the breakdown of amino-acids

The strain of *E. coli* used in this work can form, under suitable growth conditions, specific enzymes bringing about the decarboxylation of *l*(+)-arginine, *l*(+)-ornithine, *l*(+)-lysine and *l*-(−)histidine with the formation of agmatine, putrescine, cadaverine and histamine respectively. When investigated in the intact organism, the arginine decarboxylase has an optimum *p*H lying between 4·0 and 4·5; ornithine decarboxylase at 5·0; lysine decarboxylase between 4·5 and 5·0, and histidine decarboxylase between 4·0 and 4·5. The conditions of growth under which these enzymes are formed have been studied in detail [Gale, 1940*a*]: when growth occurs at *p*H 7 or higher the resulting organisms possess little or no decarboxylase activities, the enzymes appearing as the growth *p*H falls and reaching very high activities when the growth *p*H is of the order of 5 or less (Q_{CO_2} ornithine = 500; arginine = 330; lysine = 200; histidine = 30). The presence of the specific substrate during growth at an acid reaction is probably necessary for the formation of these enzymes. This result has now been obtained with many organisms [Gale, 1940*a*; 1941] and it appears certain that amino-acid decarboxylases are formed by bacteria only in response to an acid growth environment. Accordingly we began this investigation by studying the variation with growth *p*H of the amino-acid deaminases of *E. coli*. As the decarboxylases of this organism are inhibited by high growth temperatures, we have grown all cultures at 27° so that the results should be comparable with those previously obtained with the decarboxylases. The experimental temperature used, except where otherwise stated, was 30°.

dl-*Alanine deaminase*. *E. coli* possesses an enzyme which deaminates *dl*-alanine with the formation of pyruvic acid and NH_3 [Gale, 1940*b*]. The enzyme formation is inhibited by anaerobic growth conditions so that cultures were grown in this case in liquid media in Roux bottles lying on their sides. The activity of the washed suspensions was determined by shaking aerobically with *dl*-alanine solution and buffer, taking samples at intervals for NH_3 determination by the method of Parnas; details as described by Stephenson & Gale [1937*a*]. Activities are expressed as $Q_{NH_3} = \mu$l. ammonia-N liberated per hr. per mg. dry weight of organism. In the first place it was necessary to determine the optimum activity *p*H which has been recorded previously as 7·5 [Stephenson & Gale, 1937*a*]. However it was found that the optimum activity *p*H varies with the nature of the buffer in which the experiment is carried out. Fig. 2*b* shows that the optimum *p*H lies between 7·0 and 7·5 if determinations are carried out in the presence of phosphate, but if borate or veronal buffers are used, then the optimum *p*H lies near 9·5, while the activity at *p*H 7 is less in veronal than in phosphate. The figures quoted were obtained with a

culture grown at *p*H 7 but the curves obtained with cultures grown at other *p*H values differ only in magnitude. Since the enzyme thus exhibits two *p*H optima, estimations of the activities of cultures have been made in every case at *p*H 7 in phosphate and at *p*H 9·5 in borate. Fig. 2*a* shows the variation in Q_{NH_3} so determined for cultures grown in

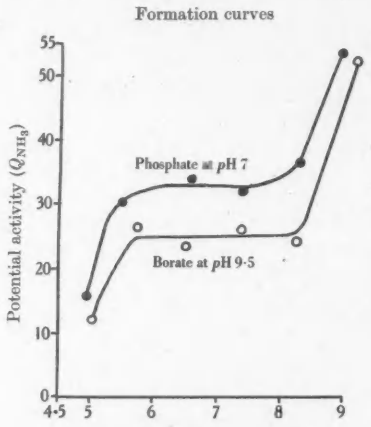

Fig. 2*a*.

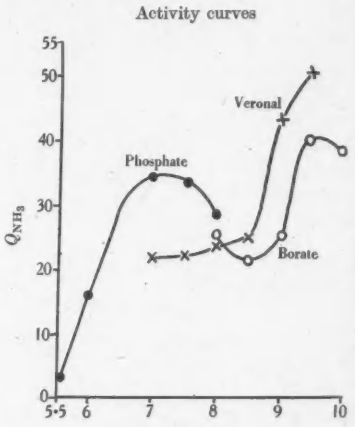

Fig. 2*b*.

Fig. 2. Alanine deaminase. (*a*) Variation of activity (Q_{NH_3}) with growth *p*H. (*b*) Activity-*p*H curves in various buffers: culture grown at *p*H 7.

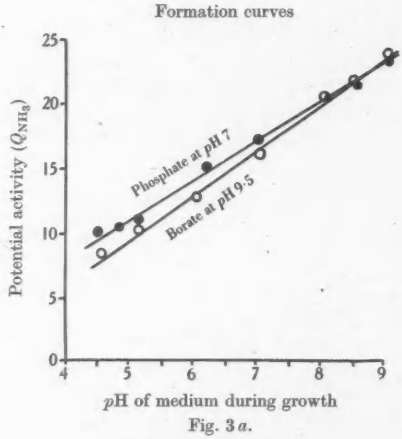

Fig. 3*a*.

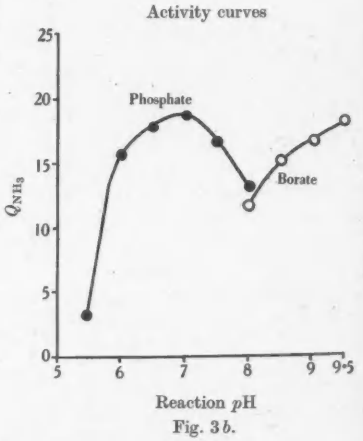

Fig. 3*b*.

Fig. 3. Glutamic acid deaminase. (*a*) Variation of activity (Q_{NH_3}) with growth *p*H. (*b*) Activity-*p*H curves in phosphate and borate: culture grown at *p*H 7.

media adjusted to *p*H values between 4·5 and 9; the growth *p*H values recorded are the average of the initial and final *p*H of the medium in each case (Table 1). Whichever buffer is used we get a curve of the same shape showing little alanine deaminase formation in acid growth conditions, a plateau over the centre of the range and increasing enzyme formation as the *p*H moves from 8 to the alkaline limit of growth. Determination of the

optimum activity *p*H for cultures grown at *p*H 5 or 9 shows that this has not shifted with growth *p*H whether estimated in phosphate or borate.

l(+)-*Glutamic acid deaminase.* The deamination of glutamic acid by *E. coli* is carried out by a reversible dehydrogenase-coenzyme system similar to that found in animal and

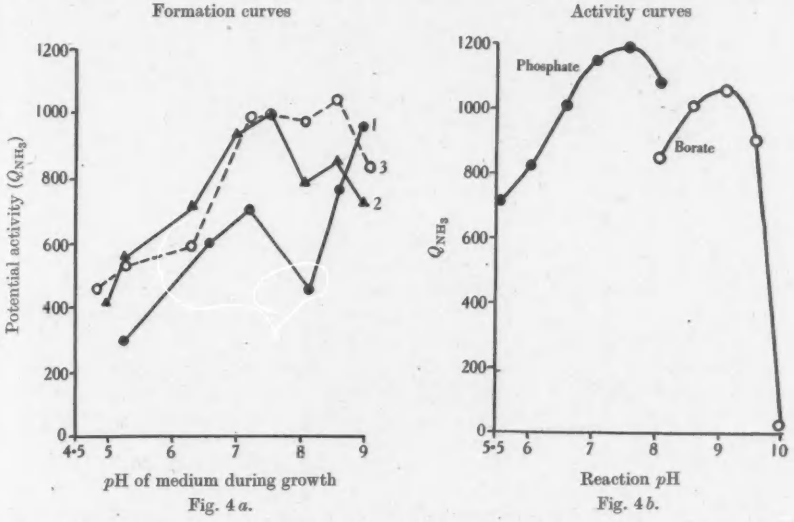

Fig. 4. Serine deaminase. (*a*) Variation of activity (Q_{NH_3}) with growth *p*H. 1, 14 hr. culture: estimations in phosphate at *p*H 7·5. 2, 16 hr. culture: estimations in phosphate at *p*H 7·5. 3, 16 hr. culture: estimations in borate at *p*H 9. (*b*) Activity-*p*H curves in phosphate and borate for culture grown at *p*H 7·5.

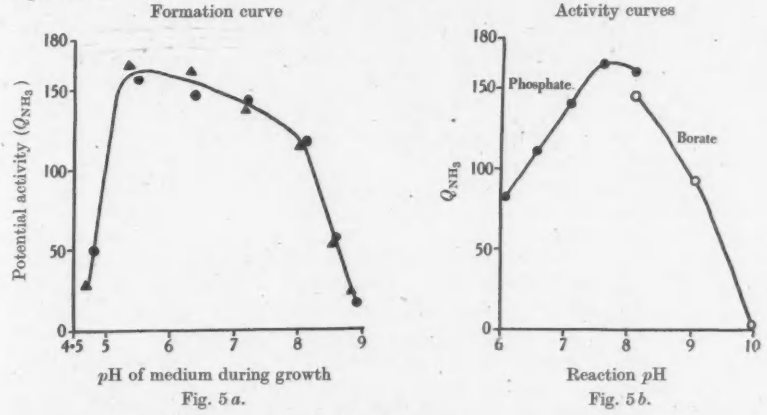

Fig. 5. Aspartic acid deaminase. (*a*) Variation of activity (Q_{NH_3}) estimated in phosphate at *p*H 7·5 with growth *p*H—2 series of estimations. (*b*) Activity-*p*H curve estimated with culture grown at *p*H 7.

plant cells [Adler, Hellstrom, Gunther & Euler, 1938]. Estimations of activity were carried out in a manner similar to that used for alanine deaminase except that cultures were grown in flasks, as semi-anaerobic conditions do not inhibit enzyme formation in this case [Stephenson & Gale, 1937*a*]. Fig. 3*b* shows that the value of the optimum

activity *p*H for a culture grown at *p*H 7·5 again varies with the nature of the buffer used. Accordingly estimations have been made for all cultures at *p*H 7 in phosphate and at *p*H 9·5 in borate. Fig. 3*a* shows the variation of the activity, so determined, of cultures grown at various *p*H values. In this case we get a linear increase in enzyme formation as the growth *p*H moves from 5 to 9, whether the activities are determined in phosphate or borate.

dl-*Serine deaminase.* Gale & Stephenson [1938] showed that *E. coli* can deaminate *dl*-serine anaerobically, the formation of the deaminase being favoured by anaerobic growth conditions. Estimations of the activity in this case are complicated by the fact that the activity of a culture falls rapidly once the cells are made up into washed suspension. This loss of activity can be checked if the cells are washed and kept in the presence of *M*/100 phosphate and *M*/200 formate, which acts as a reducing agent. In the present work, cultures have been grown for 14 or 16 hr. at 27°, centrifuged out of

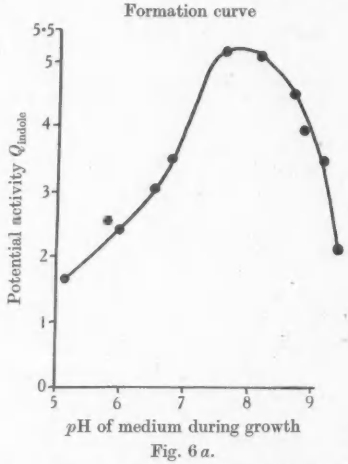

Fig. 6*a*.

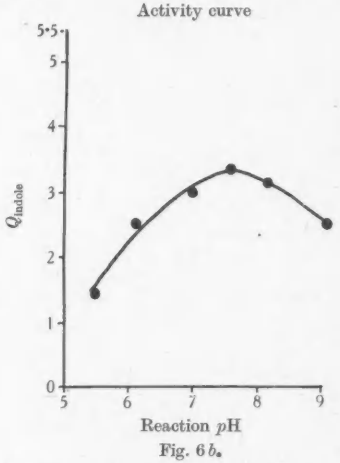

Fig. 6*b*.

Fig. 6. Tryptophanase. (*a*) Variation of activity (Q_{indole}) estimated at *p*H 7·5 with growth *p*H. (*b*) Activity-*p*H curve estimated with culture grown at *p*H 7.

culture, washed once in *M*/100 phosphate *p*H 7·5 and *M*/200 formate, made up in *M*/100 phosphate and the activities estimated as rapidly as possible after preparation of the suspensions. In this way decay of the activities should have been minimized. Fig. 4*b* shows that the *p*H optimum again shows a variation with the nature of the buffer used but in this case the enzyme is almost inactive at an external *p*H of 10 in borate, and shows two marked optima at *p*H 7·5 in phosphate and at *p*H 9 in borate. The formation curve varies slightly according to the age of the culture but exhibits two optima, one at a growth *p*H of 7·5 and another at a growth *p*H of 8·5 or 9 according to the age of the culture. The shape of the curves is the same whether they are determined in phosphate or borate and resembles that which would be obtained by combination of the two activity curves. The significance of these curves is discussed later.

l(−)-*Aspartic acid.* Quastel & Woolf [1926] found a reversible enzyme which deaminates aspartic acid anaerobically to fumaric acid in *E. coli*. This enzyme is not affected by the presence of toluene while Gale [1938] showed that *E. coli* contains at least two enzymes capable of the anaerobic deamination of aspartic acid, one being stable to toluene treatment and the other being completely inhibited under such conditions. The unstable aspartase II fraction requires the presence of a coenzyme replaceable *in vitro* by adenosine: if the activity of washed suspensions is measured aerobically

a linear rate of NH_3 evolution is obtained, but anaerobically the rate falls off rapidly with time. For the present estimations, suspensions were shaken aerobically with *l*(−)-aspartate and NH_3 estimated as usual [Gale, 1938]. The results given in Figs. 5*a* and 5*b* represent the total activities of the suspensions towards aspartic acid under such conditions. Attempts were made to separate the enzymes involved by toluene treatment but it was found that the response varied with the growth *p*H so that the activity of suspensions grown at *p*H 7–9 was greatly decreased by toluene but that of organisms grown at *p*H 5–7 was actually increased. It is obvious that such a method cannot be used to differentiate the enzymes in intact suspensions for this type of work. 'Aspartase' gives activity and formation curves of quite different shape from those obtained with the other deaminases so far studied: in this case the activity curve, Fig. 5*b*, exhibits a single optimum at *p*H 7 with no significant difference at *p*H 8 whether the activity at that *p*H is determined in phosphate or borate. The formation curve, Fig. 5*a*, shows that 'aspartase' activity is inhibited by growth on the alkaline side of *p*H 8 or at very acid values less than 5. Over the growth *p*H range 5–8, maximum formation of the enzyme takes place at the acid end of the range, the curve then showing a linear decrease in formation until the rapid fall sets in beyond *p*H 8. The formation of this deamination system is thus favoured by moderately acid growth conditions.

l(−)-*Tryptophan*. Tryptophan is degraded to indole under strongly aerobic conditions by *E. coli* and the enzyme system involved has been named 'tryptophanase' although it is realized that this is not a single enzyme [Happold & Hoyle, 1935; Woods, 1935]. The first step in the degradation has not yet been identified although Krebs, Hafez & Eggleston [1942] have recently produced evidence that *o*-aminophenylethyl alcohol is an intermediate in the production of indole. For the purpose of this work, 'tryptophanase' activity has been estimated by the simplified technique described by Fildes [1938] and activity is expressed as $Q_{\text{indole}} = \mu$l. indole produced/hr./mg. dry weight of organism. The activity—*p*H curve (Fig. 6*b*) is fairly flat as might be expected for a complex system, and has an optimum at 7·5, which *p*H has been used for the determination of the activities of all cultures. The formation of the enzyme system is greatest when the growth *p*H lies between 7·5 and 8·0 (Fig. 6*a*) and is markedly inhibited by acid or strongly alkaline growth conditions.

It is a reasonable assumption that enzymes acting upon amphoteric substrates are themselves of an amphoteric nature and it might be expected that such substances should vary in activity with the *p*H at which they are formed and so we next turned our attention to certain enzymes acting upon substrates of a non-amphoteric nature.

Enzymes concerned in the degradation of non-amphoteric substrates

The technique employed throughout this section of the work was (1) to determine the optimum activity *p*H for the enzyme concerned using a culture grown at *p*H 7, (2) using this optimum activity *p*H, to determine the activities of cultures grown at *p*H values throughout the range 4·5–9·0, (3) to redetermine the optimum activity *p*H with cultures grown at the ends of the range and at any other growth *p*H of obvious interest, and (4) in selected cases, to determine the activities of the range of cultures each at the *p*H at which it was grown instead of at the optimum activity *p*H. In the description of the work, the following terms will be employed:

Optimum activity p*H*: that *p*H at which the enzyme activity in any one culture is an optimum. This we have found experimentally does not vary with growth *p*H.

Potential activity: the activity of a culture determined at the optimum activity *p*H for the enzyme concerned.

Effective activity: the activity of a culture determined at the *p*H at which growth occurred as distinct from the optimum activity *p*H, and representing that portion of the potential activity which would actually be effective during growth in culture.

In the determination of the optimum activity *p*H, tests were made over the *p*H range 4·0–10·0 using *M*/10 phthalate, *M*/15 phosphate and *M*/20 borate buffers; to check the possibility of activity varying with the nature of the buffer, these were made to overlap; phthalate and phosphate at 5·5, phosphate and borate at 8·0. In no case described below was there any evidence of the optimum activity *p*H varying with the nature of the buffer employed.

The enzymes studied fall into two groups according to the type of variation which their formation in the cell displays with growth *p*H: in group I the loss in effective activity of the enzyme as the growth *p*H deviates from the optimum activity *p*H is compensated by an increased formation of enzyme per cell, so that the effective activity in culture is constant whatever the growth *p*H; while in group II this compensatory formation does not occur, or only to a restricted degree, while the growth *p*H is in the vicinity of the optimum activity *p*H and the enzyme is formed best when the growth *p*H approaches the value of the optimum activity *p*H.

Group I

Formic dehydrogenase. This enzyme is one of the most active of the dehydrogenases of *E. coli* and has an exceptionally high affinity for its substrate [Stickland, 1929]. The dehydrogenase reacts directly with methylene blue without the intervention of coenzyme I or II and reacts with O_2 through the cytochrome system of the bacterial cell [Gale, 1939]. Fig. 7*b* shows that the optimum activity *p*H is approximately 6·0 whether the activity is determined by the methylene blue or O_2 uptake method, and is independent of the growth *p*H. When the potential activity of cultures grown at various *p*H values is determined, we get a curve of the shape shown in Fig. 7*a* whether the estimations are made with methylene blue reduction or O_2 consumption in presence of the substrate; this shows that the formation of the enzyme is minimal when the growth *p*H coincides with the optimum activity *p*H. As the growth *p*H deviates from the value of the optimum activity *p*H, a given quantity of enzyme loses efficiency owing to that deviation, but in the cell this loss of efficiency is compensated by an increased formation of enzyme. Consequently if we estimate the effective activity of the cultures, we find that this is roughly constant throughout the growth range.

Alcohol dehydrogenase. Still [1940] has shown that *E. coli* can oxidize ethyl alcohol by means of alcohol dehydrogenase which reacts with methylene blue in the presence of coenzyme I. The enzyme can be studied as a single system if semicarbazide is present to trap the acetaldehyde and so prevent further oxidation. Activities were accordingly estimated by the methylene blue technique using Thunberg tubes with 1 ml. buffer, 1 ml. bacterial suspension, 0·5 ml. *M*/10 semicarbazide in the main compartment and 0·1 ml. 0·5% methylene blue with 0·2 ml. 50% ethyl alcohol (water in controls) in the hollow stopper. After equilibration, the contents of the stopper were tipped into the main tube and the time noted for the blue colour to disappear. Activities are expressed as $Q_{MB} = \mu$l. O_2 equivalent to amount of methylene blue reduced/hr./mg. dry weight of organism and figures are corrected for the values of the blank controls. Still [1940] noted that the addition of coenzyme to suspensions of intact cells did not significantly affect the activities, indicating that the enzyme within the cell is fully saturated with coenzyme; we confirmed this finding. Fig. 8*b* shows that the optimum activity *p*H falls at approximately 8 whatever the growth *p*H of the culture used, while the formation curve (Fig. 8*a*) has a minimum position when the growth *p*H coincides with the optimum activity *p*H. The potential activity thus shows full compensation for the deviation of the growth *p*H from the optimum activity *p*H, with the result that the effective activity is once more constant throughout the growth range.

Catalase. The catalase activity of suspensions of *E. coli* is so high that special measures have to be adopted for its measurement to attain any degree of accuracy and we are

indebted to Dr Hartree of the Molteno Institute for advice in this matter. The activity has been measured by the use of Warburg manometers containing 1 ml. bacterial suspension and 1·8 ml. buffer in the main compartment, and 0·2 ml. 3 vol. H_2O_2 in the side bulb. The amount of organism found to be satisfactory is 0·1–0·15 mg./ml. suspension.

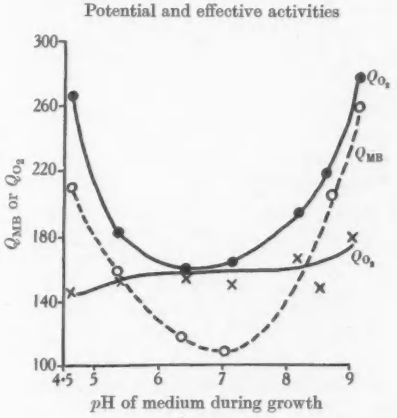

Fig. 7 *a*.

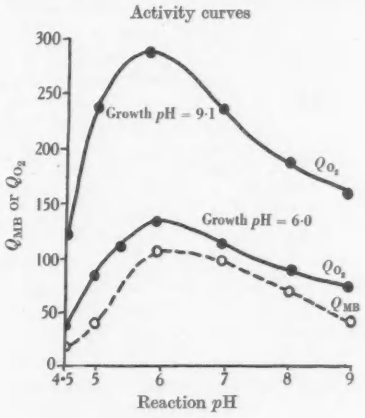

Fig. 7 *b*.

Fig. 7. Formic dehydrogenase. (*a*) Variation of activity with growth *p*H. •—• Potential activity (Q_{O_2}); ×—× effective activity (Q_{O_2}); o- - -o potential activity (Q_{MB}). (*b*) Activity-*p*H curves for cultures grown at *p*H 6·0 and 9·1.

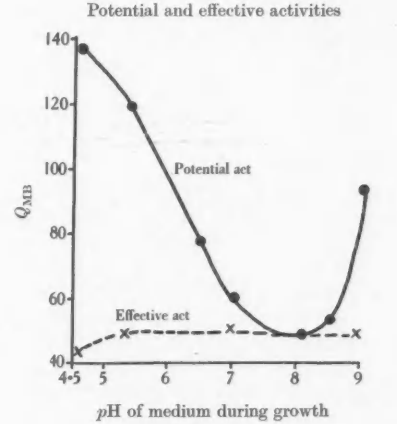

Fig. 8 *a*.

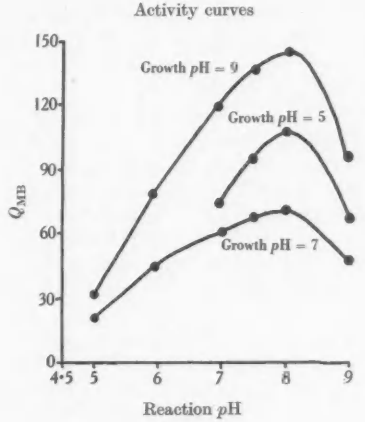

Fig. 8 *b*.

Fig. 8. Alcohol dehydrogenase. (*a*) Variation of potential activity (•—•) and effective activity (×- - -×) with growth *p*H. (*b*) Activity *p*H curves for cultures grown at *p*H 5, 7 and 9.

The manometers are shaken in a bath at room temperature (17°); the substrate is then tipped in after equilibration and readings are taken every 2 min. without stopping the shaker. The manometers are adjusted constantly by one person who takes the readings whilst another notes the times and figures. Under such conditions the enzyme is poisoned in about 20 min. but a linear rate of O_2 evolution can be followed for the first three or four 2 min. periods. Activities are given as Q_{O_2} = rate of O_2 evolution during this linear

period expressed as μl. O_2 evolved/hr./mg. dry weight organism. Fig. 9*b* shows that the optimum activity *p*H falls at about 6·5 though the variation of activity with *p*H is not very great in that region. H_2O_2 tends to decompose spontaneously in alkali but control experiments without organism showed that this effect does not vitiate the results under the experimental conditions described. The formation curve (Fig. 9*a*) is similar to those

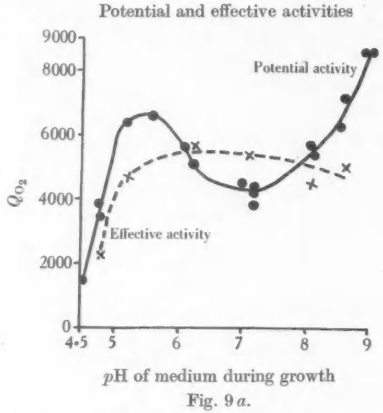

Fig. 9*a*.

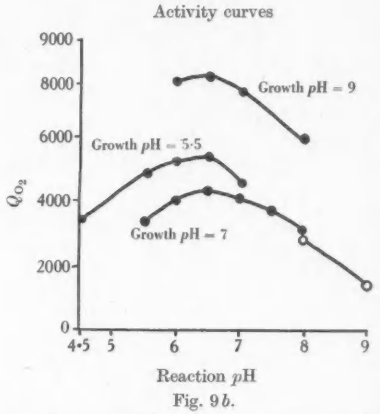

Fig. 9*b*.

Fig. 9. Catalase. (*a*) Variation of potential activity (•—•) and effective activity (×- - -×) with growth *p*H. (*b*) Activity-*p*H curves for cultures grown at *p*H 5·5, 7 and 9.

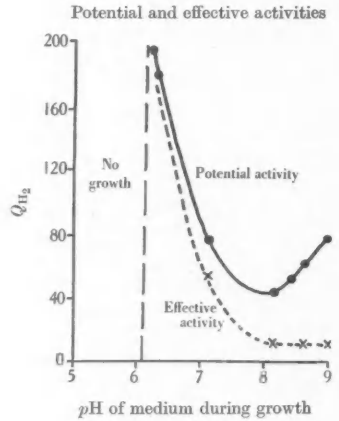

Fig. 10*a*.

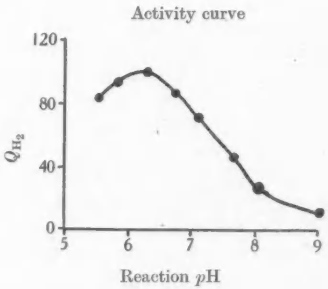

Fig. 10*b*.

Fig. 10. Formic hydrogenlyase. (*a*) Variation of potential activity (•—•) and effective activity (×- - -×) with growth *p*H. (*b*) Activity-*p*H curve for culture grown at *p*H 7.

obtained with formic and alcohol dehydrogenases over the greater part of the growth range but the compensatory formation of the enzyme breaks down when growth takes place at *p*H values below 5·5. Consequently the effective activity is roughly constant when growth occurs from *p*H 6 to 9 but below 6 the effective activity falls rapidly.

Formic hydrogenlyase. Stephenson & Stickland [1932; 1933] found that *E. coli* grown anaerobically in the presence of formate develops an adaptive enzyme decomposing

formic acid to CO_2 and H_2. Woods [1936] showed that the action of this formic hydrogenlyase is reversible. To study the variation of the enzyme with growth *p*H, cultures were grown in the usual media to which 0·5 % sodium formate was added. The addition of formate results in a decreased crop of organism and at medium *p*H values $<6{\cdot}2$ no growth will take place in its presence even when the concentration is reduced to 0·2 %. The activity of the enzyme was estimated in Warburg manometers containing 1 ml. bacterial suspension 3–4 mg. dry weight, 1·5 ml. buffer in the main compartment and 0·5 ml. *M*/30 Na formate in the side bulb (water in controls) with 0·2 ml. 10 % NaOH in the centre pot. The manometers were filled with N_2 gas freed from traces of O_2 by passage over heated copper. After equilibration, the formate was tipped in and the steady rate of H_2 evolution measured; activities are expressed as Q_{H_2}. The optimum activity *p*H is approximately 6·3 (Fig. 10*b*) and the variation of the potential activity with growth *p*H gives a unique curve (Fig. 10*a*). In this case we get minimal formation of the enzyme at a growth *p*H of approximately 8 and a rapidly increasing formation of the enzyme as the growth *p*H falls. The potential activity is greatest at the edge of the growth range where the formate becomes too inhibitory for growth to occur. The effective activity shows a similar type of variation reaching a constant low level when the growth *p*H falls below 8. It appears as though the organism attempts to remove the inhibitory substance by increased enzyme formation where the substrate is most toxic. This variation is a special case in which the formation curve bears no relation to the activity curve.

Group II

Hydrogenase. Suspensions of *E. coli* can activate molecular H_2 to reduce methylene blue by the enzyme hydrogenase [Stephenson & Stickland, 1931]. To investigate this activity, 1 ml. bacterial suspension, 3–4 mg. dry weight, and 1 ml. buffer are shaken with 0·1 ml. 0·5 % methylene blue in Thunberg tubes filled with H_2 and the time noted for the dye to be completely reduced. Fig. 11*b* shows that the optimum activity *p*H is approximately 6 but the curve obtained for the variation of potential activity with growth *p*H is completely different from those obtained with the group I enzymes. In this case the potential activity is least at the ends of the growth range and greatest at a growth *p*H of 8. Thus the optimum formation *p*H does not coincide with the optimum activity *p*H. If we determine the effective activity (Fig. 11*a*) we see that the potential activity is such that the loss of enzyme efficiency as the growth *p*H deviates from the optimum activity *p*H is compensated to some degree over the central part of the growth range only, with the result that the effective activity is roughly constant over a growth *p*H range of 6–7 but falls rapidly outside that range and is almost insignificant at the ends of the complete growth range.

Succinic dehydrogenase. Figs. 11*a* and 11*b* include the curves for succinic dehydrogenase. The activity has been determined by the methylene blue technique as before but in this case the Q_{MB} values do not correspond to the activities of a single enzyme. As in the case of hydrogenase the potential activity shows a restricted degree of *p*H compensation over the central part of the growth range only.

Glucozymase. Glucozymase is a constitutive enzyme of *E. coli* whose formation is roughly doubled by the presence of glucose during growth [Stephenson & Gale, 1937*b*]. To avoid difficulties arising over the value of the *p*H during growth in glucose, the constitutive part of the enzyme only has been studied, growth taking place in the absence of glucose. Activities were studied by estimating the disappearance of glucose incubated anaerobically in the presence of bacterial suspensions and buffer, using the ceric sulphate method of glucose estimation [Giragossintz, Davidson & Kirk, 1936] adapted for the present work along lines similar to those devised by Trim [1938]: activities are expressed as $Q_{\text{glucose}} = \mu$l. glucose removed/hr./mg. dry weight organism. Figs. 12*a* and 12*b* show

that the optimum activity *p*H is approximately 6 and the potential activity is greatest when the growth *p*H lies between 7·5 and 8 so that compensation occurs over the middle of the growth range only.

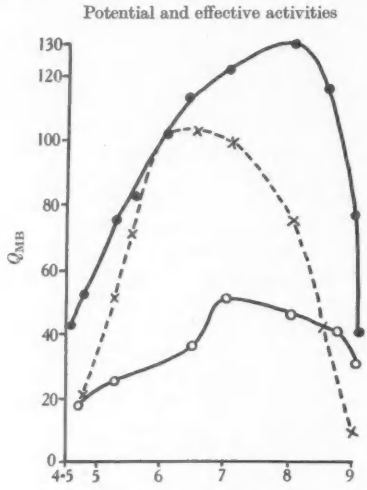

Fig. 11 *a*.

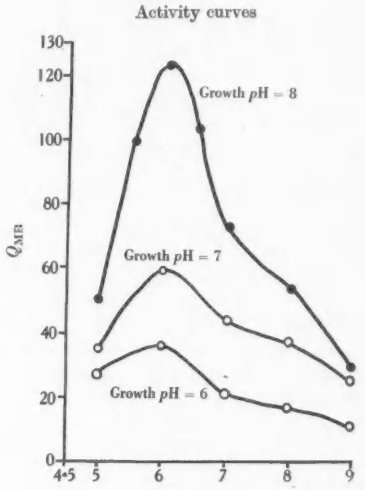

Fig. 11 *b*.

Fig. 11. Hydrogenase and succinic dehydrogenase. (*a*) Variation of activity with growth *p*H. •—• Potential activity: hydrogenase. o—o Potential activity: succinic dehydrogenase. ×- - -× Effective activity: hydrogenase. (*b*) Activity-*p*H curves for hydrogenase •—• and succinic dehydrogenase o—o.

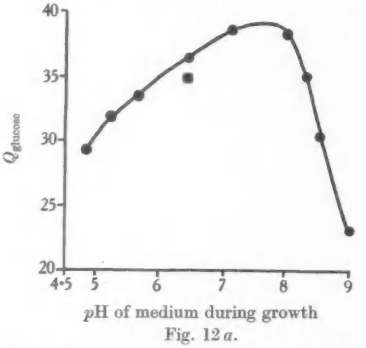

Fig. 12 *a*.

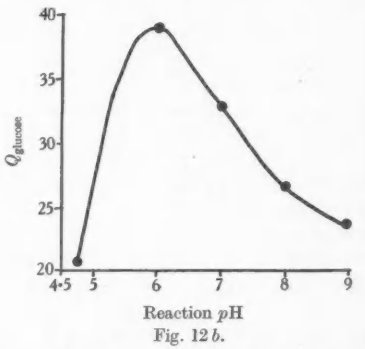

Fig. 12 *b*.

Fig. 12. Glucozymase. (*a*) Variation of activity with growth *p*H. $Q_{glucose}$ = µl. glucose disappearing/hr./mg. dry weight organism. (*b*) Activity-*p*H curve for culture grown at *p*H 7.

Variation of potential activities of washed suspensions in buffer

The variation in potential activity of certain enzymes so far described has occurred during growth at various medium *p*H values of the organisms containing them. The question arises whether similar variations can occur without growth by keeping the washed suspensions in buffers at various values within the growth range. To test this, we estimated the activities of two group I enzymes, formic and alcohol dehydrogenases, and

one group II enzyme, hydrogenase. A washed suspension of *E. coli* was prepared from a culture grown at *p*H 6 and the initial activities determined as described above. Four tubes were then set up, each containing 3 ml. washed suspension of 4 mg. dry weight organism/ml., and 1 ml. of buffer at *p*H 4·5, 6·0, 8·0 and 9·0 respectively added one to each tube. The tubes were then left at 27° for 2 hr., the organisms spun down in each and made up again in water to 4 ml. suspension. The formic, alcohol and hydrogenase activities were then determined, at the optimum activity *p*H in each case, for each suspension. Table 3 shows that there has been a general decrease in all activities but that the loss of activity has been greatest for all three enzymes at *p*H 4·5 and 9 so that there is no evidence of a compensatory formation of enzyme, as discussed above, in the absence of growth.

Table 3. *Variation of activities on standing in buffer*

		*p*H of buffer during 2 hr. at 27°				
Enzyme	*Q* unit	4·5	6	8	9	Initial activity
Formic dehydrogenase	O_2	50	54	44	29	110
Alcohol dehydrogenase	M.B.	13	41	36	24	63
Hydrogenase	M.B.	8	47	37	32	100

Absolute nature of the variation of potential activity with growth p*H*

Before attempting to place any interpretation upon these results it is necessary to determine whether the variations in potential activity described ('formation curves') represent alterations in enzyme content of the cells with growth *p*H, or varying degrees of enzyme-substrate accessibility in cells of constant enzyme content. In the absence, at present, of any satisfactory method of disintegrating *E. coli* cells and of making quantitative cell-free preparations of many of the enzymes mentioned, we have carried out analogous experiments with *M. lysodeikticus* to investigate this point. Fleming [1922] found that *M. lysodeikticus* is rapidly lysed when incubated with lysozyme, which is readily obtained by the addition to the suspension of a few drops of egg-white solution in saline. Penrose & Quastel [1930] found that this lysis involves the destruction of many enzymic activities such as formic and alcohol dehydrogenases, while other enzymes such as catalase, urease and fumarase remain unimpaired or even enhanced in activity after lysis. The organism grows thickly on the surface of nutrient agar but to obtain cultures in liquid media, in which the *p*H can be controlled, we had to adopt the measures described above. The organism will not grow in liquid media which have been adjusted to *p*H values $< 6{\cdot}5$ but is able to grow as far as *p*H 9·5 on the alkaline side. Quastel [1937] used a final concentration of 1/500 egg-white for lysis, incubating at 40° until the process was complete as judged by the disappearance of any turbidity from the suspension. Except in the case of urease we have found that such a procedure is satisfactory but allowance has to be made for wide variations in the lysozyme content of eggs.

Catalase. Estimations were carried out as described above but as *M. lysodeikticus* suspensions have about 10 times the catalase activity of equivalent *E. coli* suspensions, the amount of organism used was adjusted to 0·01 mg. dry weight; in the case of the lysed suspensions 0·001 mg. of material was sufficient. Fig. 13 shows that the potential activity exhibits a variation with growth *p*H similar to that obtained for this enzyme in *E. coli* and that the curve obtained with lysed suspensions is parallel with that obtained with the corresponding intact organisms. The catalase activity increases about 10 times on lysis of the organisms, an effect noted by Penrose & Quastel [1930], so the activities of the lysed suspensions have been reduced 10 times in Fig. 13. The constant ratio of the activity of the intact organism to that of the lysed suspension proves that the variation of potential activity with growth *p*H is due to an alteration of actual catalase content of the cells.

Urease. Penrose & Quastel [1930] state that the urease activity of *M. lysodeikticus* increases on lysis; we have been unable to confirm this but find, on the other hand, that unless lysis takes place in the presence of a high concentration ($M/24$) of urea, the urease

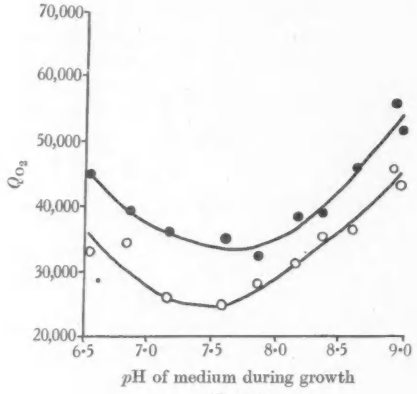

Fig. 13.

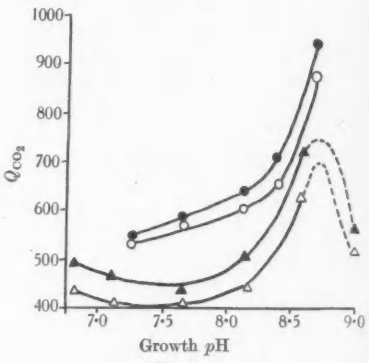

Fig. 14.

Fig. 13. Variation of potential catalase activity, estimated at *p*H 6, with growth *p*H (*M. lysodeikticus*). •—• Intact organism. o—o Lysed suspension (reduced 10 ×).

Fig. 14. Variation of potential urease activity, estimated at *p*H 6·0, with growth *p*H (*M. lysodeikticus*). 2 series. •—•, ▲—▲ Intact organism. o—o, △—△ Lysed suspension.

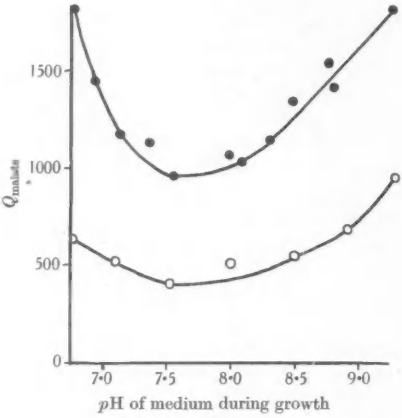

Fig. 15 *a*.

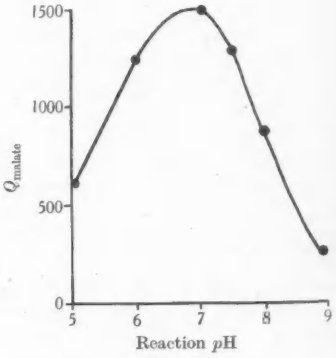

Fig. 15 *b*.

Fig. 15. Fumarase (*M. lysodeikticus*). (*a*) Variation of potential activity with growth *p*H. Q_{malate} = μl. malate formed/hr./mg. organism. •—• Lysed suspension. o—o Intact organism (increased 10 ×). (*b*) Activity-*p*H for lysed suspension of organism grown at *p*H 7.

activity is mostly destroyed during lysis. We eventually determined the activity manometrically, using $M/15$ phosphate buffer *p*H 6 (1·2 ml.), 1 ml. bacterial suspension of dry weight 1 mg., 0·5 ml. $M/4$ urea, in the main cup and 0·3 ml. egg-white solution in buffer in the side bulb. The manometers were shaken in a bath at 37° and readings taken every 5 min. until the rate of CO_2 evolution was steady in each case; then the egg-white was

tipped in from the side bulb and readings resumed after a 5 min. interval during which lysis took place. After lysis the rate of CO_2 evolution remains steady for 20–30 min. or until the buffer is exhausted. When cultures were grown as usual in urea-free media, the results were very irregular from day to day and in the response to growth *p*H but these irregularities ceased when 0·5% urea was incorporated in the growth medium. Fig. 14 shows that the potential activity exhibits a fully compensated type of variation (group I type) with growth *p*H although the compensation tends to break down when the growth *p*H is higher than 9. The activity of the lysed suspensions is slightly lower than that of the intact organism suspensions but there is a constant ratio between these activities for each culture, indicating that we are dealing with variations in actual enzyme content.

Fumarase. Penrose & Quastel [1930] state that the fumarase activity of *M. lysodeikticus* increases greatly on lysis. Since fumarase activity is estimated by the formation of malic acid from fumaric acid, the smaller activity of the intact organism is probably explained by the removal of malic acid in this case by enzymes which are destroyed during lysis. Fumarase activity was estimated as follows: sufficient organism can be obtained from one Roux bottle to provide 7 ml. of suspension of dry weight 6–8 mg./ml.; this was mixed with 7 ml. buffer and 7 ml. *M*/2 fumarate and the whole shaken at 37°. At 30 and 60 min. after mixing, 10 ml. were withdrawn from each container and added to 10 ml. 14·2% ammonium molybdate solution followed by the addition of 1 ml. glacial acetic acid. The samples were left in the dark for 2–3 hr., the precipitate filtered off and malic acid estimated polarimetrically by the method of Auerbach & Kruger [1923]; activities expressed as $Q_{\text{malate}} = \mu$l. malate formed/hr./mg. dry weight organism. Figs. 15*a* and 15*b* show that fumarase has a fully compensated group I type of variation with growth *p*H with minimal formation occurring when the growth *p*H coincides approximately with the optimum activity *p*H. The variation is shown equally well with the lysed suspension or the apparently less active intact organism.

M. lysodeikticus does not appear to possess aspartase, alanine deaminase or glucozymase when tested under the usual conditions for these enzymes.

The absolute nature of the variation of enzyme formation with growth *p*H is shown also in the case of certain extracellular enzymes of *Cl. welchii* studied by Gale & van Heyningen [1942]: the production of hyaluronidase shows a typical group I variation with growth *p*H while that of α and θ toxins shows a group II variation.

Discussion

The enzymes investigated in the course of this work can be divided into two main groups according to the type of variation they exhibit with alteration of the *p*H of the medium during growth.

Group I: those enzymes for which a compensatory formation takes place as the growth *p*H deviates from the optimum activity *p*H so that the effective activity remains approximately constant whatever the growth *p*H.

Group II: those enzymes which are formed best when the growth *p*H approximates to the optimum activity *p*H. In some cases where the optimum activity *p*H is near 7, a compensatory formation takes place over the middle of the growth range so that the effective activity is constant in this region but falls off rapidly towards the extremes of the range.

The enzymes of *E. coli* and *M. lysodeikticus* so far studied are distributed between the two groups as follows:

Group I	Group II	
Formic dehydrogenase	Hydrogenase	Serine deaminase
Alcohol dehydrogenase	Succinic dehydrogenase	(Aspartase)
Catalase	Glucozymase	Arginine decarboxylase
Urease	Tryptophanase	Ornithine decarboxylase
Fumarase	Alanine deaminase	Lysine decarboxylase
(Formic hydrogenlyase)	Glutamic acid deaminase	Histidine decarboxylase

The effective activities have not been determined in the case of the deaminases owing to the change in their activities with the nature of the buffers used. Kocholaty & Hoogerheide [1938] give an activity-*p*H curve for alanine dehydrogenase of *Cl. sporogenes* which shows two optima, at *p*H 7 and 9·5, when the whole range is covered with phosphate buffers. We find that phosphate systems are such unsatisfactory buffering agents for *p*H values higher than 8 that such figures must be accepted with reserve. With alanine deaminase of *E. coli* the activity-*p*H curve determined in veronal buffers appears to have only one optimum (Fig. 2*b*) and that at approximately 9·5. If these deaminases can be regarded as mechanisms complementary to the decarboxylases, which have optimum activity *p*H values ranging from 2·5 to 5·5, then the alkaline values obtained in veronal and borate could be regarded as the true optimum activity *p*H figures, whilst phosphate has a 'salt effect' resulting in a shift of the optimum. We cannot solve this problem satisfactorily until the enzymes concerned have been obtained in a cell-free state. Whatever may be the true interpretation, the formation of the serine, alanine and glutamic acid deaminases is greatest when the *p*H of the external growth environment approaches this more alkaline optimum activity *p*H, whilst the alanine and serine curves show signs of an increased formation also at the optimum activity *p*H of 7·0–7·5 found in phosphate. These considerations justify the inclusion of these deaminases in group II. Aspartase is unusual in that its potential activity displays some considerable degree of compensatory formation for growth *p*H values 5–7 but the shape of the curve (Fig. 5*a*) outside these values places the enzyme in group II. Aspartic acid would seem to be the only amino-acid of those studied which can be utilized by the cell as a source of N at acid growth *p*H values.

The amino-acid decarboxylases appear to be adaptive enzymes which are formed only when the organism grows under acid conditions, when the growth *p*H approaches the optimum activity *p*H. In such an acid medium, the carboxyl groups of the amino-acids are presumably undissociated, while in an alkaline medium the —COOH groups will be ionized and the —NH_2 groups undissociated. Thus we find that the organism grown under alkaline conditions can no longer produce decarboxylases but attacks certain amino-acids by deamination. Both decarboxylases and deaminases can therefore be looked upon as adaptive enzymes where the specific substrate stimulating enzyme formation is the undissociated form of the molecule involved. Likewise they may be neutralization mechanisms, the decarboxylases being formed in response to an acid medium and by their action tending to neutralize that acidity, and certain deaminases being formed under opposite conditions and having a neutralizing effect on alkaline growth conditions. That such neutralizing mechanisms do exist is shown by the drift of the *p*H of unbuffered media towards neutrality during growth of the organisms. The action of these mechanisms will so modify the *p*H of the internal environment of the cell that this will not be subject to such wide variations as shown by the growth range of *p*H measured in the external environment. A probable consequence of this is that, for enzymes which are most active under strongly acid or alkaline conditions, the optimum activity *p*H determined with intact cells and measured in the external environment will differ from the true figure for the cell-free enzyme by an amount determined by the capacity of the cell to control the *p*H of its internal environment. We intend to publish data demonstrating this point later.

Formic hydrogenlyase is placed in group I on account of the general shape of its potential activity curve (Fig. 10*a*) although the minimal enzyme formation does not take place when the growth *p*H coincides with the optimum activity *p*H. Formic acid is obviously toxic to *E. coli* under acid conditions of growth and formic hydrogenlyase acts by removing the inhibitory substance under these conditions. It is obvious that the enzymes of group I play a different part in the economy of the bacterial cell from those of group II, since their formation is so controlled that they should be equally effective under all conditions of growth. Their activity would seem to be essential to the existence

of the cell. Formic hydrogenlyase is formed by the cell only under anaerobic growth conditions, in response to the presence of formic acid, when it may be assumed that the formic dehydrogenase will be inefficient. Likewise it has been recorded that formic dehydrogenase has an unusually high affinity for its substrate [Stickland, 1929] and undergoes little variation with the age of the culture [Wooldridge, Knox & Glass, 1936]. Thus the cell has evolved mechanisms for dealing with this substance under almost all conceivable growth conditions. The substrates of other enzymes in group I such as urea, alcohol and H_2O_2 are known to be toxic to bacteria and so it would seem that some at any rate of these group I enzymes are protective or detoxication mechanisms. It is too early to generalize as yet: there is little evidence that fumaric acid is toxic to bacteria. Further, the strain differences in the distribution of these enzymes are difficult to explain unless it may be that the organisms produce these enzymes to protect themselves from the toxic products of their normal metabolism.

We see from these results in general that bacteria react to an alteration in their external environment by an alteration in their enzymic constitution. The changes appear to involve two principles: (1) an attempt to counter the adverse external change and (2) an attempt to maintain essential activities at a constant value. In other words, bacteria react to a change in their external environment in such a way that the resultant change in their internal environment is at a minimum.

Summary

1. *E. coli* can grow in casein digest adjusted to any *p*H between 4·5 and 9.

2. Change in the external *p*H during growth is followed by an alteration in the enzyme content of the cells. The enzymes can be divided into two groups according to the type of their variation with growth *p*H.

Group I: those enzymes whose formation undergoes a variation so that their activity per cell is constant whatever the medium *p*H. This means that the potential activity of the cells in respect of these enzymes increases as the growth *p*H deviates from their optimum activity *p*H.

Group II: those enzymes whose formation is greatest when the growth *p*H approaches their optimum activity *p*H. In cases where the optimum activity *p*H lies between 6–8, a small degree of compensatory formation takes place over the centre of the growth range so that the effective activity is constant over that part of the range but falls off rapidly towards the extremes.

3. The group I enzymes appear to have a protective function in the cell in that they remove inhibitory substances, e.g. urease, catalase, formic dehydrogenase and hydrogenlyase, etc.

4. Group II enzymes include the amino-acid decarboxylases and deaminases so that the former are produced in response to an acid growth environment and the latter in response to an alkaline growth *p*H. In this manner they act as neutralization mechanisms.

5. These variations in enzyme content do not occur in the absence of growth.

6. Similar variations in the enzymes of *M. lysodeikticus* are not affected in nature by lysis of the cell suspensions with lysozyme; therefore the variations observed are due to alterations in actual enzyme content of the cells concerned.

7. In general, a change in the external *p*H is followed by an alteration in the enzymic constitution of the cells such that (1) an attempt is made to counter the external change and (2) certain essential activities are maintained at a constant level.

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70. The Influence of the Presence of Glucose during Growth on the Enzymic Activities of *Escherichia coli*: Comparison of the Effect with that Produced by Fermentation Acids

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(*Received 29 June 1942*)

Kendall & Farmer [1912; 1913] showed that in growing cultures of a number of different bacterial species the ammonia liberated from protein digests decreases or even disappears when carbohydrate is present. Kendall [1922] suggested that this effect was due to the protein-sparing action of carbohydrate. Berman & Rettger [1918] showed that the excretion of protease by *P. vulgaris* is also inhibited by the presence of carbohydrate in the medium but since the effect is less marked with an organism such as *B. subtilis* which produces little acid from glucose, they suggested that the effect was due to the production of acid from fermentation of the carbohydrate. The addition of buffer to the medium decreased the inhibitory effect of glucose and this supports the theory that the effect is one of *p*H. Raistrick & Clark [1921] pointed out that the growth of many bacterial species is much heavier in the presence of glucose in the medium and suggested that the greater yield of cell-N accounts for some of the missing ammonia-N, but this factor is insufficient to explain the discrepancy in most cases. Happold & Hoyle [1935] made a non-viable preparation of 'tryptophanase' which would bring about the aerobic degradation of tryptophan to indole and they reported [1936] that cultures of *E. coli* grown in the presence of glucose contained no tryptophanase. Fildes [1938] reported that the tryptophanase of *E. coli* can be divided into a small constitutive portion and a large adaptive portion, and that the presence of glucose during growth inhibits the formation of the adaptive portion of the enzyme. Evans, Handley & Happold [1941] claim that tryptophanase is purely adaptive and that the presence of glucose in the growth medium completely inhibits its formation as long as tyrosine or phenylalanine is also present; in the absence of these amino-acids the inhibitory effect of glucose is not complete, a result explaining the findings of Fildes [1938].

Stephenson & Gale [1937 *a*] found that the addition of glucose to washed suspensions of *E. coli* grown in the absence of glucose has no significant effect on the activity of the glycine, alanine and glutamic acid deaminases, but if the organism is grown in the presence of 2% glucose, the resulting suspensions have only 10–20% of the deaminase activities of those grown in its absence. The effect of glucose is therefore not on the course of the enzyme action after growth is complete but inhibits the formation of the deaminases during growth. This result was later obtained with *dl*-serine deaminase [Gale & Stephenson, 1938] and aspartase [Gale, 1938]. In order to check the *p*H effect of the fermentation of glucose during growth, chalk was mixed with the medium and constantly stirred throughout the growth period, without effect on the inhibitory action of glucose on the deaminase formation. The effect was not due to anaerobiosis produced by fermentation gases, for bubbling the medium with oxygen did not affect the inhibition. Gale [1940; 1941], investigating the conditions under which bacteria produce amino-acid decarboxylases, found that the action of glucose in the medium in promoting the formation of these enzymes could be explained entirely by the low *p*H produced by fermentation during growth. This raised the question whether the addition of chalk to the medium in the

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previous work was an efficient method of controlling the medium *p*H, particularly within the fermenting cell. The present communication is an attempt to settle this problem of whether the effect of glucose in the medium can be attributed to the action of the acidity formed during its fermentation.

Most of the investigations of this glucose effect have been restricted in the past to enzymes concerned in the metabolism of amino-acids, probably owing to the emphasis placed on the 'protein-sparing' hypothesis, and as we were determining the action of growth *p*H on the enzymic activities in general [Gale & Epps, 1942] we decided to extend the observations to include cultures grown in the presence of glucose.

Methods. The methods of estimating the various activities dealt with below have all been outlined in the previous paper by Gale & Epps [1942]. Growth took place in tryptic digest of casein adjusted initially to *p*H 7 and to 5 (the final *p*H attained by *E. coli* in 2% glucose broth) as described before and, in addition, in broth containing 2% glucose and adjusted initially to *p*H 7. Growth took place at 27° and the final *p*H was estimated in all cases.

Experimental

Table 1 gives the activities of cultures of *E. coli* grown in plain broth at *p*H 7 and 5 and in 2% glucose broth in which the *p*H altered from 7 to 5·2 (average) during growth. The variation of activity with *p*H during growth has already been dealt with in the previous paper. If the effect of the glucose in the medium is due to the *p*H produced by its fermentation, then the activities of the cultures grown in glucose should approximate to those of cultures grown at *p*H 5. Table 1 shows that in certain cases such as those of hydrogenase and catalase, and arginine, lysine and histidine decarboxylases the difference in the activities of the *p*H 7 and glucose cultures can be ascribed to the alteration in *p*H during growth. In all the other cases quoted, with the exception of glucozymase, glucose has an inhibitory effect on the activity greater than any effect that can be ascribed to *p*H. This is particularly the case with formic and alcohol dehydrogenases where an acid growth medium produces an increased potential activity compared with growth at *p*H 7 while the action of glucose is markedly inhibitory. Glucozymase is a special case in that the presence of glucose increases the formation of this enzyme while acid growth conditions decrease it; thus the adaptive increase in glucozymase noted by Stephenson & Gale [1937*b*] is a true substrate effect.

Table 1. *Potential activities of* E. coli *when grown in casein digest adjusted to* p*H 7 or 5, and containing 2% glucose*

Final *p*H in glucose medium after growth = 5·2 (average).

Enzyme	*Q* unit	Potential activity in medium: At *p*H 7	At *p*H 5	2% glucose	Glucose effect
Hydrogenase	M.B.	240	136	146	None
Catalase	O_2	4200	6360	6310	,,
Arginine decarboxylase	CO_2	2	338	272	,,
Lysine decarboxylase	CO_2	53	194	198	,,
Histidine decarboxylase	CO_2	3	26	33	,,
Ornithine decarboxylase	CO_2	47	560	48	Inhibitory
Alanine deaminase	NH_3	32	4	1·7	,,
Glutamic acid deaminase	NH_3	12	3	1·2	,,
Aspartase (total)	NH_3	127	247	15	,,
Serine deaminase	NH_3	855	656	167	,,
Tryptophanase	Indole	5·4	1·6	0·25	,,
Alcohol dehydrogenase	M.B.	52	179	44	,,
Succinic dehydrogenase	M.B.	43	23	9	,,
Formic dehydrogenase	M.B.	110	138	58	,,
Formic hydrogenlyase	H_2	75	>200	139	,,
Glucozymase	Glucose	38·5	31	77	Stimulatory

Table 1 shows that the inhibitory action of glucose on the formation of enzymes is not confined to amino-acid deaminases, although the effect is marked with these systems, particularly in the case of aspartase where the culture grown at *p*H 5 has approximately double the activity of that grown at *p*H 7 while the glucose culture has about 1/9 of that activity. To confirm that the glucose effect cannot be explained in terms of *p*H, a culture of *E. coli* was grown in 2% glucose broth adjusted to *p*H 7 and the *p*H was maintained at this value throughout the growth period by the addition of sterile NaOH solution to neutralize fermentation acids. The *p*H was measured by means of a hydrogen electrode immersed in the medium, the whole apparatus being the same as that described by Gale & van Heyningen [1942]. The activities of three cultures (1) in plain broth at *p*H 7, (2) in 2% glucose broth maintained at *p*H 7 and (3) in 2% glucose broth with *p*H uncontrolled, were then determined and the results set out in Table 2 show that the inhibitory action of glucose in the medium is the same, for the deaminases tested, whether the fermentation acidity be neutralized or not.

Table 2. *Potential activities of* E. coli *when grown in glucose*

Enzyme	*Q* unit	Potential activity when grown in: No glucose at *p*H 7	2% glucose *p*H uncontrolled	2% glucose *p*H 7 maintained
Alanine deaminase	NH_3	43	12	15
Glutamic deaminase	NH_3	13	5	5
Aspartase (total)	NH_3	127	15	9
Aspartase (toluene-treated)	NH_3	45	18	19
Dry weight *coli*/ml. medium		0·27 mg.	0·71 mg.	1·31 mg.

The results so far described have been obtained after a single subcultivation in the presence of glucose. Next we tested whether it is possible to train the deaminase activities to maintain the lower level by serial subcultivation in glucose broth. 100 ml. of plain broth were inoculated with *E. coli* and incubated overnight. Then 100 ml. 2% glucose broth were inoculated from this culture and the remainder spun off for the determination of the aspartase activity of the washed suspension. This culture was then subcultivated five times in 2% glucose broth and the activity determined after each incubation. The fifth glucose culture was then subcultured into plain broth. Table 3 shows that there is

Table 3. *Effect of repeated subcultivation in 2% glucose broth*

Growth medium	Hr. of growth	Q_{NH_3} (aspartic)	Growth medium	Hr. of growth	Q_{NH_3} (aspartic)
Plain broth	16	143	2% glucose broth (4)	7	33
2% glucose broth (1)	16	24	2% glucose broth (5)	16	24
2% glucose broth (2)	7	32	Plain broth (1)	12	135
2% glucose broth (3)	16	25			

no progressive loss of activity on consecutive 'passages' through glucose broth and that the organism regains its normal aspartase activity immediately on cultivation in the absence of glucose.

Results with Micrococcus lysodeikticus

Quastel [1937] investigated the effect of the presence of glucose in the growth medium upon certain enzymic activities of *M. lysodeikticus*. He claimed that glucose stimulates the formation of urease and suppresses that of catalase and fumarase, the estimations being carried out on lysed suspensions. Quastel grew his cultures on the surface of peptone-agar; we have grown our cultures in casein-digest broth as described previously but have obtained different results under these conditions. When *M. lysodeikticus* is grown in liquid medium, the addition of 1% glucose does not cause any alteration in the *p*H during

growth and we have been unable to find any significant activity towards glucose of washed suspensions prepared from organisms so cultivated. The addition of glucose to the suspension does not cause any significant increase in the O_2 consumption measured manometrically and the anaerobic $Q_{glucose}$ measured as described for glucozymase [Gale & Epps, 1942] is less than 2. When the dry weight of the suspensions and the activities of these enzymes are determined by the methods described in the previous paper [Gale & Epps, 1942] we get results of the order shown in Table 4 showing no significant effect upon the catalase, urease and fumarase activities due to growth in the presence of glucose, whether the activities are determined with intact organism or lysed suspensions. The difference between our findings and those of Quastel may be explained in the case of catalase by the much longer experimental period used by him: we have shown that the enzyme is poisoned within 15–20 min. of the addition of the H_2O_2 and that a rate of enzymic decomposition can only be measured during the first 4–6 min. after mixing at room temperature, so that Quastel's estimation of the O_2 evolved after 2 hr. at 38° is no measure of the enzymic activity. Also we have found that the urease activity of this organism is largely destroyed by lysis unless the lysis takes place in the presence of urea; it is not possible to calculate the Q_{CO_2} from Quastel's figures, since the weight of organism is not given, but the volumes of CO_2 quoted (87–176 μl. in 2 hr.) suggest that the activity measured is the activity which remains left after lysis in the absence of substrate. Likewise we have found that the urease activity of *M. lysodeikticus* can vary greatly unless growth takes place in the presence of urea.

Table 4. *Effect of growth in glucose on activities of* M. lysodeikticus

			Potential activity when grown in	
Enzyme	Q unit	Organism	Broth at pH 7	1% glucose broth at pH 7
Catalase	O_2	Intact	51,000	50,000
		Lysed	330,000	320,000
Urease	CO_2	Intact	300	298
		Lysed	193	194
Fumarase	Malate	Lysed	1,420	1,490

Addition of glucose has no significant effect on pH of medium during growth.

Summary

1. The presence of glucose in the medium during the growth of *E. coli* suppresses the formation of certain enzymes; the degree of inhibition is greater than, or bears no relation to, the effect produced by growth in a medium adjusted to the final pH produced in the glucose medium by fermentation acids.

2. Neutralization of fermentation acids during growth in glucose does not alter the degree of inhibition of deaminase formation produced by the glucose.

3. The reduction of the activity of certain enzymes as a result of growth in glucose is not a permanent change in the enzyme constitution of the cell as it is removed immediately growth takes place in the absence of fermentable carbohydrate.

4. The presence of glucose in the liquid growth medium has no effect upon the urease, catalase and fumarase activities of *M. lysodeikticus*.

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71. The Effect of the *p*H and the Presence of Glucose during Growth on the Production of α and θ Toxins and Hyaluronidase by *Clostridium welchii*

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When grown in a suitable medium *Cl. welchii*, type A, excretes α and θ toxins, hyaluronidase and proteolytic enzymes. α toxin has been shown by Macfarlane & Knight [1941] to be closely associated, and probably identical, with an enzyme, lecithinase; θ toxin acts as a haemolysin when in a reduced state [Todd, 1941], but the mechanism of its action has not yet been elucidated. The excretion and adaptive nature of hyaluronidase have been studied by McClean & Hale [1941]. When grown in an acid medium or in the presence of a high concentration (2%) of glucose, the organism develops histidine decarboxylase, thus giving rise to histamine in the medium [Gale, 1941]. This enzyme is only formed when growth occurs at *p*H $< 5{\cdot}5$ and the object of this communication is to establish (1) the optimum medium *p*H conditions for the production of α and θ toxins and hyaluronidase, and (2) the part played by glucose in the medium in the production of these substances. The amino-acid metabolism of *Cl. welchii* has been studied by Woods & Trim [1942].

Methods

The organism used for the whole of this work was *Cl. welchii*, type A, strain S 107. The medium used for growth of the organism was that described by Macfarlane & Knight [1941] with and without 0·45% glucose, with the exception that 1·5% 'Allenbury's Beef Extract' was substituted for the sodium sulphate protein-free extract of horse-meat. Preliminary experiments indicated that it was not possible to control the *p*H of the medium by the addition of buffer solutions, since the presence of phthalate or borate inhibits the growth of the organism. It was therefore necessary to control the *p*H during growth by the addition of sterile acid or alkali as required and the following apparatus was devised. 500 ml. medium were sterilized by filtration through a Ford G.S. filter and transferred with sterile precautions to a three-necked flask. A hydrogen electrode, sterilized in alcohol, was introduced, together with one arm of a sterile agar-bridge, through one neck of the flask. A delivery tube reaching to the bottom of the flask and bent over outside to form a siphon acted as a sampling device and passed through the second neck. The third neck was fitted with a wide glass tube reaching to the surface of the medium and plugged with a sterile bung: sterile *N* NaOH or HCl was added through this tube as required by means of a Pasteur pipette and the medium stirred after each addition by a stream of N_2 passed to the bottom of the flask through a third tube in this neck. The *p*H of the medium was measured by means of the hydrogen electrode connected to a valve-potentiometer. The flask was incubated in a bath maintained at 37° and inoculated from a 12-hr. meat-tube culture of the organism. At suitable intervals after inoculation the medium was stirred and approximately 10 ml. samples withdrawn by the siphon. The dry weight of organism/ml. medium was estimated by the use of a photo-electric turbidimeter previously calibrated for the organism concerned [Clifton, Mueller & Rogers, 1935]. The logarithm of the dry weight was plotted against the age of the culture and the generation time, representing the time taken for the dry weight of organism to become doubled during the logarithmic phase of growth, calculated from the linear portion of the curve.

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The sample was then clarified by adjusting to neutrality, adding a knife-point of kieselguhr and centrifuging down the kieselguhr and the adsorbed organism. The following estimations were carried out on the clear supernatant fluid.

1. α toxin was estimated by the method of van Heyningen [1941*a*]; activity expressed as E.U./ml. medium or mg. dry weight of organism.

2. θ toxin was estimated by measuring the time in which an aliquot of a given sample brings about complete haemolysis of a red blood cell suspension under standard conditions. When necessary the samples were treated with thiolacetic acid to reduce the toxin. The suspensions of red cells and the dilutions of toxin are made in isotonic 0·0665 *M* phosphate buffer *p*H 6·5 so that the α toxin is completely inhibited [van Heyningen, 1941*b*]. The sheep red cell suspension used is approximately 1·5%, and is made up daily from a stock standard approximately 25% suspension, the preparation of which has already been described [van Heyningen, 1941*b*]. A flat (tintometer) cell, 4 mm. wide, is suspended in a water bath at 38° against the inside of the front glass wall of the thermostat tank. Immediately behind the cell, and attached to it by means of a rubber band, is a microscope slide with a fine vertical line scratched on it. Behind this device is an electric light bulb. 1 ml. of an appropriate dilution of the toxin is pipetted into the cell, and when it has reached 38° (1 min.) 1 ml. of the 1·5% red blood cell suspension, previously warmed to 38°, is added. The time in which the *fine detail* of the scratch on the microscope slide becomes visible through the haemolysing red cell suspension is taken as the time for complete haemolysis. This end-point can be observed fairly easily with practice; when the haemolysis time is 100 sec. the end-point can be determined within ± 3 sec., and the percentage error appears to be the same over a range of 50–300 sec. From the time taken for complete haemolysis the corresponding number of units of θ toxin in the aliquot used can be determined by reference to a previously determined standard activity curve. One θ unit is arbitrarily defined as the amount of θ toxin which will bring about complete haemolysis of a standard, approximately 0·75%, suspension of sheep red cells at 38° in 100 sec. The error of this method appears to be within $\pm 5\%$.

3. Toluene was added to clear samples which were sent to Dr McClean for estimations of the hyaluronidase titre. This enzyme was titrated by the mucin clot prevention (M.C.P.) test which depends upon the determination of the highest dilution of the sample which, when incubated for a standard time with a substrate mixture composed of a hyaluronic acid-protein complex, will destroy the power of this mixture to form a typical 'mucin clot' on the addition of acetic acid. A complete description of this test together with a study of its correlation with other methods of estimating hyaluronidase and diffusing activity is being prepared for publication. Most of the 24-hr. samples, which contained the highest titre of enzyme, were also titrated by the viscosimetric method [McClean & Hale, 1941]; there was good agreement in the relative values obtained by these two methods. Hyaluronidase activity is expressed as M.C.P. titre/ml. of medium or per mg. dry weight of organism.

All estimations other than hyaluronidase determinations were made on samples taken from the first appearance of turbidity and at half-hour intervals from then until growth ceased. Hyaluronidase estimations were made on samples taken (1) during the middle of the growth phase, (2) at the end of the period of active growth and (3) 24 hr. after inoculation. Activities have been calculated in all cases per ml. medium and per mg. dry weight of organism; experience shows that the latter is the most consistent way of expressing activities in cultures in which the total crop may vary slightly from culture to culture.

Experimental

Fig. 1*a* shows the variation of the generation time with the *p*H of the medium in which growth occurs. *Cl. welchii* will grow in the medium described at any *p*H between 5 and 8·5, although the generation time is too long below *p*H 5·5, and the total crop of organisms

then obtained too small, for useful experimental work. Glucose slightly decreases the generation time in most cases, its greatest effect being in the alkaline growth range. Fig. 1*b* shows the variation of the total crop (i.e. maximum dry weight observed) obtained at various growth *p*H values; glucose markedly stimulates the production of organism.

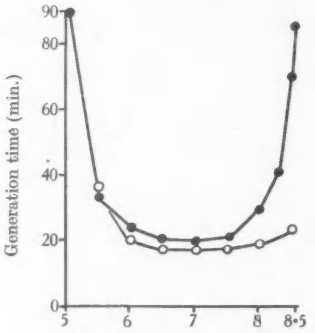

Fig. 1*a*.

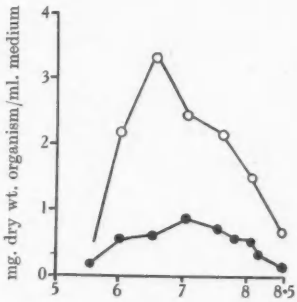

Fig. 1*b*.

Fig. 1. (*a*) Variation of generation time with growth *p*H (*Cl. welchii*). (*b*) Variation of total crop with growth *p*H. •—• No glucose. o—o 0·45% glucose.

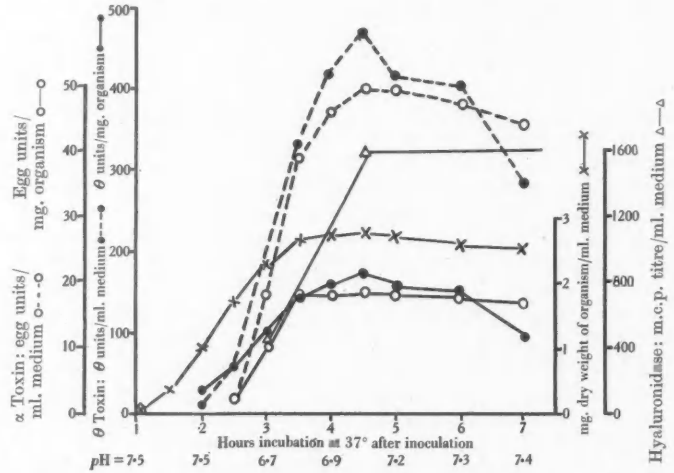

Fig. 2. Variation with age of culture (*Cl. welchii* S 107) in medium containing 0·45 % glucose at an initial *p*H of 7·5. Growth temp. = 37°.

Fig. 2 shows the variation of toxin and hyaluronidase production with the age of the culture. It can be seen that θ toxin appears in the medium a short time before the α toxin and that both toxin and hyaluronidase activities of young cultures are small; they increase as growth proceeds and reach a maximum at about the time that active cell division ceases. After this stage the activities of both α and θ toxins decrease in the medium but hyaluronidase activity remains constant or, in some cases, even increases.

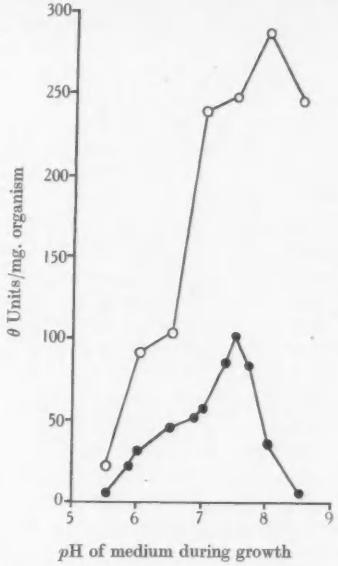

Fig. 3. Variation of θ toxin production per mg. organism with growth *p*H.
●—● No glucose. ○—○ 0·45 % glucose present.

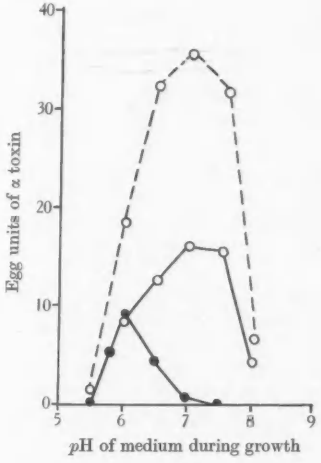

Fig. 4.

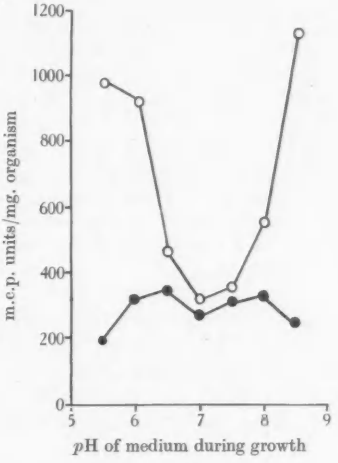

Fig. 5.

Fig. 4. Variation of α toxin production with growth *p*H. ●—● Egg units/mg. organism: no glucose. ○—○ Egg units/mg. organism: 0·45 % glucose. ○- - -○ Egg units/ml. medium: 0·45 % glucose.

Fig. 5. Variation of hyaluronidase production per mg. organism with growth *p*H. ●—● No glucose. ○—○ 0·45 % glucose.

The case quoted represents the conditions holding in a medium containing 0·45 % glucose with the *p*H initially adjusted to 7·5, no attempt being made to control the *p*H during growth. This case represents the usual conditions of growth used for toxin production, but the general shape of the curves for toxin, hyaluronidase and organism production against time remains the same for all conditions of growth studied. The figures quoted below for toxin content of media are calculated for the time of maximum toxin production in each case, while the hyaluronidase activities are calculated from the activity of the 24-hr. sample divided by the maximum dry weight of organism recorded during growth.

Two series of experiments along these lines were carried out: first the formation of toxin, etc., was studied in glucose-free media adjusted to *p*H values from 5·5 to 8·5 with intervals of 0·5 *p*H unit; secondly, the series was repeated with the addition in each case of 0·45 % glucose to the medium. The *p*H was maintained at a steady value in each experiment; in the absence of glucose this is not difficult as the drift of *p*H towards neutrality due to the metabolic activities of the growing organism is easily countered, but when glucose is present in the medium it is necessary to add about 1 ml. of *N* NaOH every 5 or 10 min. during the period of rapid growth in order to neutralize the acid produced by fermentation. Fig. 3 shows the production of θ toxin per mg. dry weight of organism at the various medium *p*H values tested: in the absence of glucose there is a marked production optimum at a growth *p*H of 7·5; the presence of glucose markedly increases the yield of toxin at all growth *p*H values and results in an apparent shift of the optimum production *p*H to 8·0. Fig. 4 shows the corresponding variations of α toxin: in the absence of glucose α toxin production is negligible except for growth *p*H values between 5·5 and 6·5; the presence of glucose results in an entirely different curve, with a greatly increased toxin production over the range 6–8, and with an optimum at 7·0–7·5. The growth *p*H for optimum production of α toxin thus shifts from 6·0 in the absence of glucose to 7 or 7·5 in its presence. The yield of α toxin/ml. medium shows an optimum when the medium *p*H is stabilized at 7·0.

Fig. 5 shows the variation of hyaluronidase production/mg. dry weight of organism with medium *p*H. In the presence of glucose there is a minimum production of hyaluronidase at a growth *p*H of 7 and the production increases rapidly as the growth *p*H value becomes either acid or alkaline, so that the highest yields per mg. dry weight of organism are obtained at the extremes of the growth range. In the absence of glucose, the much smaller growth makes the hyaluronidase activity too small to be estimated with accuracy but it appears probable that the shape of the production-*p*H curve is similar to that obtained in the presence of glucose, especially around the centre part of the growth range. The activities estimated for a growth *p*H of 7 with and without glucose indicate that glucose does not significantly stimulate the production of hyaluronidase per mg. dry weight of organism.

Finally, the estimations for media containing glucose and adjusted to initial *p*H values of 8·5, 7·5, 6·5 and 5·5 were repeated, but in these cases the *p*H was allowed to alter without control. The results are set out in Table 1 and indicate that an initial *p*H of 7·5 gives the highest production of both α and θ toxins estimated per ml. medium. It appears that the yield of α toxin when the *p*H is uncontrolled is slightly higher than when the *p*H is stabilized, but insufficient experiments have been carried out to be certain on this point.

p*H stability of* α *and* θ *toxins.* α and θ toxins, in the crude bacterial filtrate, appear to be quite stable in the *p*H range used in these growth experiments. 10 ml. aliquots of a toxic filtrate were adjusted to the *p*H values, determined with the glass electrode, shown in Table 2, and left at 22° for 5 hr. After that time, the aliquots were adjusted to neutrality, made up to 20 ml. and their α and θ activities determined.

Table 1. *Production of α and θ toxins and hyaluronidase by* Cl. welchii *growing in the presence of glucose with varying initial* p*H*

Initial *p*H	Age of culture hr.	*p*H	mg. organism/ml. of medium	α toxin		θ toxin		Hyaluronidase	
				E.U./ml.	E.U./mg.	θ U./ml.	θ U./mg.	Titre/ml.	Titre/mg.
8·5	6½	8·08	0·650	4·2	6·5	57	79	80	120
	8½	7·40	1·400	33	23·5	468	334	—	—
	24	—	—	—	—	—	—	1280	910
7·5	3	6·75	1·700	18	10·6	170	100	400	235
	4½	7·11	2·700	50	18·5	475	176	1600	592
	24	—	—	—	—	—	—	1600	592
6·5	2½	6·39	0·575	1·6	2·8	16	28	80	140
	5	6·36	3·37	40	12	220	66	640	190
	24	—	—	—	—	—	—	1280	380
5·5	4½	5·25	0·102	1·1	11	1·8	17	10	98
	5½	5·42	0·05	3·3	?	2·2	?	10	?
	24	—	—	—	—	—	—	100	?

In each case readings are given for (1) middle of growth phase, (2) end of period of active growth, and (3) 24 hr. after inoculation.

Table 2. p*H stability of α and θ toxins*

*p*H	Percentage of maximum activity after 5 hr. at 22°		*p*H	Percentage of maximum activity after 5 hr. at 22°	
	α	θ		α	θ
2·5	33	28	7·3	100	100
3·3	38	42	8·5	100	78
4·1	72	85	9·1	81	61
5·3	96	100	9·8	60	49
6·3	96	98			

Discussion

Figs. 3 and 4 indicate that glucose is toxigenic for α and θ toxins; this action of glucose cannot be ascribed to changes in medium *p*H produced by fermentation acid, since the *p*H of the medium has been controlled in all the experiments involved and, furthermore, toxin production is not favoured by an acid growth medium. Since α toxin is not produced at growth *p*H values >7 in the absence of glucose it is possible to produce a culture filtrate containing θ toxin free of α toxin by growing the organism at *p*H 7·5 in the absence of glucose.

It is interesting to note that the maximum production of α and θ toxins takes place when the growth *p*H is in the neighbourhood of 7. This *p*H is also the optimum for the growth of the organism. Hyaluronidase production, on the other hand, is lowest in this range.

Summary

Cl. welchii, type A, strain S 107 has been grown in a peptone-salt-beef extract medium with and without glucose; the *p*H has been stabilized in each case at various values within the growth range 5·5–8·5. Under these conditions the production of α and θ toxins and hyaluronidase per mg. dry weight of organism varies with the age of the culture, being small in young cultures and increasing to a maximum at the time when active cell division ceases. In the absence of glucose, θ toxin production per mg. dry weight of organism is maximum for a growth *p*H 7·5; glucose increases the yield of toxin at all *p*H values and shifts the growth *p*H for maximum production to 8·0. In the absence of glucose α toxin is produced only when the medium *p*H lies between 5·5 and 7·0 with a maximum production at *p*H 6·0; in the presence of glucose the maximum production takes place at medium *p*H values 7·0–7·5. The formation of hyaluronidase per mg. dry weight of organism is least when the medium *p*H is 7·0 and increases as the medium *p*H becomes more acid or alkaline.

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72. Intercellular Hormones

4. Further Observations Regarding the Mechanism of the Production and Release of Proliferation-promoting Factors by Injured Cells

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When cells of yeast or animal tissues are injured by agents such as lethal ultra-violet which do not lead directly to rupture of the membrane or to rapid death, proliferation-promoting factors other than dead-cell disintegration products or autolysis products appear in the intercellular fluids [Loofbourow, Dwyer *et al.* 1938; 1940; 1941; Davidson, 1940]. Evidence that such proliferation-promoting factors (intercellular wound hormones) are released by living injured cells rests primarily on the following observations [Loofbourow, Dwyer & Cronin, 1941]: (1) slow injury leads to higher potencies of intercellular fluids than rapid killing, (2) the potency of the fluids increases rapidly before appreciable mortality is noted, (3) the potency is greater when cells are injured in a physiologically favourable suspension medium, (4) active factors appear in suspensions of cells subjected to anaerobiosis without killing the cells, and (5) cells killed quickly by grinding or boiling and then subjected to injurious ultra-violet yield less potent preparations than cells injured slowly by ultra-violet prior to quick killing by grinding or boiling. It has been concluded from such data that the proliferation-promoting activity of intercellular fluids from slowly-injured cells is attributable to either (*a*) release of materials already present in the cell as a result of increased membrane permeability, (*b*) release of materials synthesized in the living cell as a response to injury, or (*c*) both of these processes. The further studies presented here were directed at a more complete elucidation of the mechanism involved in this phenomenon.

Experimental methods and results

S. cerevisiae Fleischmann, bakers' strain, was washed repeatedly and suspended in distilled H_2O at a concentration of 100 g. wet weight per litre of suspension. The suspension was divided into two equal portions, one of which was irradiated with lethal ultra-violet while the other served as a control. Following irradiation, the intercellular fluids were separated off by centrifuging and filtration through Seitz filters. Assays for proliferation-promoting activity were made on yeast cultured in Reader's medium at 30° in a roller tube device. Details of the irradiation and growth techniques have been reported [Loofbourow, Webb, Loofbourow & Abramowitz, 1942].

1. *Cell counts.* There was no significant change in haemacytometer counts throughout the course of the injury period, either in controls or irradiated suspensions as illustrated by the two examples in Table 1. This confirms similar observations by Davidson [1940], who used isotonic saline as the suspension medium. In experiments of longer duration (up to 22 hr.) at lower ultra-violet intensity levels, and employing Reader's medium, increase in cell number was noted in both controls and irradiated suspensions, but in no instance was a significant decrease in cell number, indicative of loss of cells by cytolysis, observed.

2. *Results of microscopic examinations.* No evidence of cytolysis was found on examination of the suspensions at intervals throughout the course of irradiation. After approximately 4 hr., budding of some cells was noted. The percentage of budded cells was uniformly greater in irradiated than in control suspensions (10·7 and 6·6% respectively at the end of 5 hr. in preparation 13. 12. 41).

Table 1. *Variation in cell number per ml. during course of injury by ultra-violet radiation as determined by haemacytometer counts*

Time, hr. after start of irradiation	% deviation from mean for each series			
	Prep. 8. 11. 41		Prep. 13. 12. 41	
	Non-irradiated %	Irradiated %	Non-irradiated %	Irradiated %
0	+5·5	+5·0	−6·0	− 5·7
1·5	−2·5	−4·9	—	—
3·0	—	—	+6·1	+ 1·2
5·0	—	—	+7·6	+10·4
6·5	−3·1	+4·8	—	—
8·0	+0·1	−4·9	−7·7	− 5·9

Average cell counts in all instances were approximately 10^9 cells per ml. Small buds occasionally observed attached to mother cells late in the irradiation periods were not counted as separate cells.

A larger percentage of budded than of unbudded cells in the irradiated suspensions, but not in the controls, stained with methylene blue. At the 5 hr. observation of the 13. 12. 41 irradiated preparation, approximately two-thirds of the budded cells, but only half of the unbudded cells, were stained. This might be explained by greater susceptibility of the budded cells to the lethal radiation, or it may indicate a correlation between injury and tendency to bud formation.

3. *Results of tests for presence of protein.* The intercellular fluids gave negative biuret and phosphotungstic acid precipitation tests, in agreement with previously reported results for other preparations [Loofbourow, Cook & Stimson, 1938]. Spectra of the fluids showed no maxima in the region 2700–2900 Å. characteristic of proteins, tyrosine, or tryptophan. Davidson [1940] found his extracts from irradiated cells to give a biuret reaction. Their protein nitrogen content was much less than that from similar suspensions of autoclaved cells (8·7 mg./100 ml. as compared with 75·6 mg./100 ml.) but was higher than that of control extracts (2·1 mg./100 ml.). It is possible that the discrepancies between his results and ours were due to more drastic irradiation of his preparations.

4. *Evidence regarding changes in membrane permeability.* The effects of injury and death in increasing the permeability of cell membranes are well known, and it has been suggested that this may account for the release of proliferation promoters by injured cells [Loofbourow, Dwyer & Cronin, 1941]. The following observations substantiate this view. Preliminary studies with radioactive phosphorus indicated that permeability is increased during the course of irradiation. Cells removed from the irradiated suspensions late in the irradiation period appeared shrunken, as observed by Davidson [1940]. The stained cells were generally smaller than the unstained ones. When yeast cells were irradiated continuously on the stage of a quartz ultra-violet microscope so that the same individual cells could be observed from time to time, there was a progressive decrease

Table 2. *Changes in cell volume during the progress of injury by ultra-violet radiation. Values listed are % of total suspension-volume occupied by cells*

Time, hr. after start of irradiation	Prep. 8. 11. 41		Prep. 13. 12. 41	
	Non-irradiated	Irradiated	Non-irradiated	Irradiated
0	11·5	11·5	11·0	11·0
1·0	—	—	11·5	10·5
1·5	12·0	11·0	—	—
2·0	—	—	11·5	10·5
3·0	—	—	11·5	10·5
5·0	—	—	11·5	9·0
5·5	12·0	9·0	—	—
8·0	12·0	8·5	12·0	7·5

in cell size [Loofbourow & Joyce, 1941; and unpublished data]. Finally, haematocrit determinations on preparations in distilled water showed progressive decrease in the total volume of irradiated cells as compared with an increase in the volume of control cells (Table 2).

There is evidently an increased permeability of the membranes of the injured cells, accompanied by loss therefrom of water-soluble crystalloidal substances and reduction in turgor.

5. *The possible role of direct photochemical action in the production of proliferation-promoting factors.* It has been suggested from time to time that the proliferation-promoting factors found in the intercellular fluids from ultra-violet injured cells might arise as a result of direct photochemical action. In view of the fact that the intercellular fluids from cells injured mechanically, chemically, or by subjection to CO_2 [Loofbourow & Dwyer, 1938; 1939; Loofbourow, Cook *et al.* 1939] show similar proliferation-promoting activity, direct photochemical conversion of an inactive into an active substance can hardly be considered as uniquely responsible for this type of phenomenon. Nevertheless, the possibility had to be considered that direct photochemical activation might have been involved in those instances in which ultra-violet radiation was employed as the injuring agent.

In order to determine whether such a mechanism could be considered reasonable from the point of view of energetics, the following measurements and computations were made. Radiation of longer wave-length than about 2950 Å., which is the long-wave limit of the lethal ultra-violet region, was found to be ineffective in causing the release of proliferation-promoting factors. From energy measurements with a calibrated Hanovia ultra-violet meter and transmission measurements with a monochromator and thermopile, it was determined that the absorbed energy in the region $\lambda < 2950$ Å., neglecting surface reflectance, was $4{\cdot}23 \times 10^8$ erg/ml. of suspension for the 8 hr. irradiation periods employed. The surface reflectance was calculated to be about 5% from data of Supplee & Dorcas [1934], a fraction too small to be of significance in the computations. To determine the approximate number of quanta absorbed, the energy of the quantum at 2500 Å. was used, since the source of ultra-violet was a low-pressure mercury arc having a large proportion of its energy output in the range $\lambda < 2950$ Å. concentrated in the 2537 Å. mercury resonance line. On this basis, the number of quanta absorbed per ml. was $\frac{4{\cdot}23 \times 10^8}{7{\cdot}96 \times 10^{-12}} = 5{\cdot}31 \times 10^{19}$. The growth-stimulating potency of a typical preparation was 106 growth units*/ml. greater than that of the corresponding control. Hence the number of quanta per additional growth unit was $\frac{5{\cdot}31 \times 10^{19}}{106} = 5{\cdot}0 \times 10^{17}$.

If the photochemical activation process had a quantum efficiency of 1, with no reduction in efficiency due to absorption of interfering substances, $5{\cdot}0 \times 10^{17}$ molecules of activated material would, therefore, correspond to one growth unit. If, as is more likely, the absorption of energy by interfering substances greatly reduced the overall efficiency, the number of activated molecules per growth unit would be much less. For purposes of approximation, it may be assumed that the conditions are analogous to the photochemical activation of sterols to vitamin D, which for ergosterol in solution has a quantum efficiency of about 0·2–0·5 [Marshall & Knudson, 1930] but for milk has a quantum yield of 7×10^{-6} [Ellis, Wells & Heyroth, 1941, p. 844], the overall efficiency

* Our experimentally determined potencies are based on the quantity of material which must be added to each ml. of a yeast suspension of 0·064 mg./ml. (wet weight) in Reader's medium to give a 24 hr. crop of 1·6 mg./ml. (approximately 6,250,000 cells/ml.) greater than the control crop, the cultures being grown at 30° under aerated conditions. This standard biological effect is defined as a 'growth unit'. The quantity of material in mg. or ml., necessary to give this effect having been determined, the reciprocal of this quantity then gives the number of growth units per mg. or per ml. [Loofbourow, Dwyer & Lane, 1940].

being reduced by 2×10^{-5} because of the dissipation of energy by non-activable absorbing substances in the milk. Assuming a similar factor for the effect of interfering substances in the yeast suspension, the number of activated molecules per growth unit would be $(5{\cdot}0 \times 10^{17})\ (2 \times 10^{-5}) = 1{\cdot}0 \times 10^{13}$.

Such considerations, then, lead to the deduction that if direct photochemical activation accounts for the proliferation-promoting factors, the number of activated molecules per growth unit is of the order of $1{\cdot}0 \times 10^{13}$ to $5{\cdot}0 \times 10^{17}$, the lower value being more probable.

It is now possible to determine whether the growth-stimulating activity of such an hypothetical substance would be of reasonable order of magnitude. Data of Williams, Eakin & Snell [1940], recalculated to our potency basis, and unpublished experiments of our own, indicate that pantothenic acid has a growth potency of about $4{\cdot}8 \times 10^{-5}$ mg. per growth unit under our assay conditions. This is equivalent to

$$\frac{4{\cdot}8 \times 10^{-5} \times 10^{-3}}{229} \times 6{\cdot}06 \times 10^{23} = 1{\cdot}27 \times 10^{14} \text{ molecules per growth unit.}$$

Comparing this with the computed values for the hypothetical photoactivation product, one finds for the latter a potency of the order of $2{\cdot}5 \times 10^{-3}$ to 12 times that of pantothenic acid, depending upon whether the higher or the lower of the two assumed quantum yields is employed. These are not unreasonable values, and the possibility of direct photochemical activation giving rise to the proliferation-promoting factors cannot, therefore, be ruled out by a *reductio ad absurdum* argument on the grounds of energetics alone.

The significance of these computations lies in their relation to the question of whether or not the effects observed by the ultra-violet cell-injury technique are characteristic of cell damage generally or are specifically related to ultra-violet injury. Although, as stated above, the observation of similar increases in the growth potencies and ultra-violet absorption of intercellular fluids from cells damaged in other ways indicates that the phenomenon is not specifically characteristic of ultra-violet injury, the above computations suggest the need of caution in making too broad generalizations from ultra-violet injury experiments. Lethal radiant energy is convenient and easily applied in investigating cell-damage phenomena, but the results obtained with it should be checked by other methods.

6. *Effects of monochromatic ultra-violet radiation.* A preliminary investigation of the effects of monochromatic ultra-violet radiation on wound hormone production was carried out as follows: a General Electric H-6 1-kW. high-pressure water-cooled mercury arc in a quartz envelope was used as a source with a Bausch and Lomb constant-deviation double-prism quartz monochromator. A calibrated thermopile and galvanometer served for energy measurements. The yeast employed was suspended in distilled water at a concentration of 0·873 mg. wet weight/ml. It was necessary to use this low concentration, as compared with 100 mg./ml. used in full ultra-violet preparations, in order to obtain appreciable killing in the 10 hr. irradiation period with the low ultra-violet intensities available from the monochromator system. The yeast suspensions were irradiated in a quartz cell of 2 cm. thickness by 3 cm. height and 3 cm. width, inside dimensions. The volume of irradiated and control suspensions was 12 ml. in each experiment. Both suspensions were stirred continuously with motor stirrers throughout the irradiation periods.

The effects of three wave-length regions were investigated, namely 2900–2800 Å, 2740–2560 and 2483–2417 Å. These are designated in what follows by the wave-lengths at the centres of the bands, respectively 2850, 2650 and 2450 Å. The band widths were chosen so that the same total energy ($1{\cdot}39 \times 10^7$ ergs) was absorbed in the irradiated suspension in all preparations. Immediately following irradiation, the control and irradiated suspensions were filtered through Seitz filters; thus the extraction period was uniformly 10 hr. in all instances. The results are given in Table 3.

Table 3. *Effects of monochromatic ultra-violet radiation on the production of wound hormones*

Time of irradiation 10 hr. in each instance. Total energy absorbed in 12 ml. of suspension, $1{\cdot}39 \times 10^7$ erg.

Wave-length Å.	Absorbed quanta per ml.	% dead cells* A Before irradiation	% dead cells* B After irradiation	% dead cells* B − A	% of total volume occupied by cells A Before irradiation	% of total volume occupied by cells B After irradiation	% of total volume occupied by cells B − A
2850	$1{\cdot}61 \times 10^{17}$	1	5	+ 4	0·06	0·04	−0·02
(2900–2800)	Control	1	1	—	0·06	0·07	+0·01
2650	$1{\cdot}50 \times 10^{17}$	1	41	+40	0·07	0·02	−0·05
(2740–2560)	Control	1	1	0	0·07	0·07	0
2450	$1{\cdot}34 \times 10^{17}$	1	2	+ 1	0·07	0·04	−0·03
(2483–2417)	Control	1	1	0	0·07	0·07	0

Wave-length Å.	Absorbed quanta per ml.	Potency of extracts. Growth units per ml.	Potency of extracts. Growth units per ml. Irradiation − non-irradiation	Quanta per growth unit of increased potency due to irradiation	Extinction at 2650 Å.	Extinction at 2650 Å. Irradiation − non-irradiation
2850	$1{\cdot}61 \times 10^{17}$	1·1	−0·1	—	1·6	+0·4
(2900–2800)	Control	1·2	—	—	1·2	—
2650	$1{\cdot}50 \times 10^{17}$	2·2	+1·1	$1{\cdot}36 \times 10^{17}$	3·0	+1·8
(2740–2560)	Control	1·1	—	—	1·2	—
2450	$1{\cdot}34 \times 10^{17}$	1·2	0	—	1·7	+0·2
(2483–2417)	Control	1·2	—	—	1·5	—

* Methylene blue test.

Of the three bands employed, only the 2650 Å. band yielded sufficiently potent preparations for quantitative growth assay. At this wave-length, the number of quanta per growth unit of increased potency following irradiation was $1{\cdot}36 \times 10^{17}$, which compares with $5{\cdot}0 \times 10^{17}$ reported above for full ultra-violet radiation in the range $\lambda < 2950$ Å. On an equal energy basis, the 2650 Å. band was markedly more effective than the 2850 or 2450 bands in bringing about (*a*) death of cells, (*b*) shrinkage of cell volume, (*c*) increase in growth potency of intercellular fluids, and (*d*) increased absorption of intercellular fluids at 2650 Å. The maximum lethal effectiveness of ultra-violet for yeast [Oster, 1934] and bacteria [Gates, 1930] lies near 2600 Å. For *B. coli*, Gates found maximum effectiveness at 2650 Å., a minimum at 2400 Å., and rapid decrease in effectiveness from 2650 Å. toward longer wave-lengths, the relative effect at 2850 being about the same as that at 2400 Å. Thus the preliminary results on effects of monochromatic radiation indicate a correlation between the lethal effectiveness of various wave bands and their ability to bring about the release of proliferation-promoting factors and nucleotide-like substances.

7. *Total yields from wound hormone extracts and autolysed residues.* In order to obtain more conclusive data as to whether or not synthesis of growth factors and nucleotide-like substances in the living, injured cells accompanies the loss of such substances to the intercellular fluids, growth-stimulating potencies and absorption spectra were determined on autolysates of the residues following irradiation and extraction as well as on the original extracts. 400 g., wet weight, of yeast were washed twice and suspended in distilled water to make a total volume of 4 l. The suspension was divided into two portions of 2 l. each, one of which was irradiated for 8 hr. with full ultra-violet, the other serving as a control. After irradiation, both suspensions were centrifuged, and the supernatants were decanted and filtered through Seitz E.K. filter pads. The manipulations up to this point followed precisely those previously described in detail [Loofbourow, Webb, Loofbourow & Abramowitz, 1942]. The residues from the centrifuged suspensions were set aside under

toluene and allowed to autolyse for 11 days. The autolysates were then autoclaved and centrifuged. The supernatants were decanted, filtered through Seitz E.K. filter pads, and made up to 400 ml. each with distilled water.

Absorption spectra and growth potencies were determined on the original extracts and on the filtrates from the autolysed residues. In the case of growth tests of the autolysates, difficulty was encountered in evaluating the potencies accurately because the relationship between 24 hr. growth and concentration was decidedly non-linear for both preparations. A further complication was introduced by the fact that at concentration levels higher than about 0·003 ml. of autolysate per ml. of culture, the autolysate from the irradiated residue was more potent than that from the non-irradiated, while at levels lower than the above value the autolysate from the non-irradiated residue was the more potent

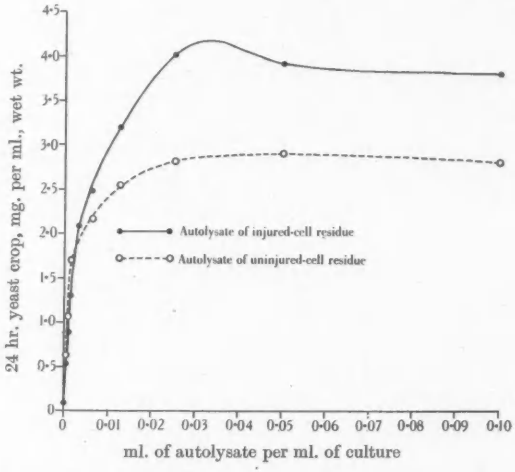

Fig. 1. Growth test of residue autolysates from injured and uninjured cells.

(Fig. 1). This difference in behaviour of the two autolysates was established by repeated assays at concentrations ranging from 0·0045 to 0·1 ml./ml. of culture. It indicates that the two autolysates differed qualitatively (i.e. in composition) as well as quantitatively.

The autolysate of the injured-cell residue was more potent than that of the non-injured cell residue throughout approximately 97% of the concentration range tested. The autolysate potencies in Table 4 are averages of those for concentrations corresponding to increased 24 hr. yeast crops of 1·6 and 2·4 mg./ml. above the control crop. This is in the range in which the autolysate from uninjured cells showed the greatest relative potency, and yields more conservative values than would be obtained by computing the potencies from higher concentration levels.

The total growth potency of extract plus residue autolysate from injured cells, as shown in Table 4, was greater than that from uninjured cells by approximately 2 to 1. This strongly suggests that synthesis of growth-stimulating materials took place in the injured cells. The potencies of the two autolysates were very nearly the same, the difference in potencies of injured- and uninjured-cell preparations lying primarily in the original intercellular fluids. Evidently resynthesis of proliferation-promoting factors just about kept pace with loss of material to the intercellular fluids throughout the course of injury.

Table 4. *Growth potency and ultra-violet absorption of wound hormone extracts and residue autolysates*

Preparation	Growth units per ml.*	Volume V, in ml.	Total growth units*	$K=\frac{1}{d}\log_{10}\frac{I_0}{I_d}$ $\lambda=2650$ Å.	KV
A. Extract from injured cells	84·7	1815	153,731	12·25	22,234
B. Autolysate of injured-cell residue	364	400	145,600	375	150,000
Totals A + B	—	—	299,331	—	172,234
C. Extract from uninjured cells	3·0	1815	5,445	2·1	3,812
D. Autolysate from uninjured-cell residue	378	400	151,200	115	46,000
Totals C + D	—	—	156,645	—	49,812

* Average values computed for two concentration levels, as explained in the text.

The spectra of all samples showed absorption curves characteristic of nucleotides, or their derivatives. The 2650 Å. extinction coefficient on a volume basis $\left(K=\frac{1}{d}\log_{10}\frac{I_0}{I_d};\ d \text{ in cm.}\right)$ is shown for each sample. Since this is proportional to the concentration of material absorbing at 2650 Å., multiplication of K by the total volumes, V, of the preparations results in values proportional to the total yields of nucleotide-like substances present. The KV value for the autolysate from injured cells was about thrice that for the autolysate from uninjured cells, while the values for the original intercellular fluids had a ratio of approximately 6 to 1 in the same direction. Hence only about 15% of the nucleotide-like material appeared in the intercellular fluids from injured cells whereas slightly more than half the growth potency was found in the intercellular fluids. It appears that, while there was marked synthesis of nucleotide-like materials in the injured cells, the greater part of the synthesized material could not permeate the cell membranes, whereas synthesized growth-stimulating materials were released into the intercellular fluids with comparative ease.

It seems clear from the data that not all of the nucleotide material was involved in growth-stimulating activity. It is conceivable that a large portion of the inactive nucleotide-like material, which failed to pass through the cell membranes, was yeast nucleic acid itself. Previous experiments have shown it to be comparatively inactive [Loofbourow, Dwyer & Lane, 1940]. Furthermore, we have obtained evidence that the yeast nucleic acid content of yeast is increased during injury by ultra-violet radiation [Loofbourow, Englert & Dwyer, 1941; Loofbourow, Webb, Loofbourow & Lisco, 1942].

The greater nucleotide-like content of irradiated cell autolysates than of non-irradiated cell autolysates is evidence against the possibility that the effect of ultra-violet irradiation of the cells is simply to break down nucleic acid-like materials already present in the cell and thus to permit their diffusion outward through the cell membrane.

Summary and conclusions

Washed suspensions of *S. cerevisiae* in distilled water were subjected to slow injury by ultra-violet radiation over periods of 8 or 10 hr. for the purpose of studying the mechanism of the release of proliferation-promoting factors by damaged cells into the intercellular fluids. The following observations resulted.

1. Inappreciable change in cell number throughout the irradiation periods indicated that the proliferation-promoting factors in the intercellular fluids were released through the membranes of grossly intact cells.

2. There was no evidence of cytolysis throughout the irradiation periods, substantiating the above conclusion.

3. Negative biuret and phosphotungstic acid tests on intercellular fluids from damaged cells indicated substantial freedom from protein, and supported the conclusion that cytolysis was negligible.

4. Preliminary studies with radioactive P have indicated an increase in membrane permeability during irradiation. This was confirmed by haematocrit determinations of cell volume, which showed an average decrease of 26% in injured-cell volume as against an increase of 8% in control suspensions. It is concluded that permeability changes play an important role in the release of proliferation-promoting factors by damaged cells.

5. It was computed from energy determinations that if the active proliferation promoter were a photochemically activated substance, it would be expected to have a potency of the order of 10^{-3} to 10 times that of pantothenic acid.

6. Preliminary studies with monochromatic wave bands centring at 2850, 2650 and 2450 Å. shows the 2650 Å. band to be the most effective in bringing about the release of proliferation-promoting factors, cell death, loss of cell volume, and increase in 2600 Å. absorption of the intercellular fluids.

7. After the removal of intercellular fluids from injured and uninjured cells, the residues were autolysed under toluene for 11 days, autoclaved, centrifuged, and the supernatants tested for growth activity and ultra-violet absorption. The total activity of original intercellular fluids plus residue autolysates was approximately twice as great for injured as for uninjured cells, whereas the activities of the autolysed residues alone were approximately the same. It is concluded that resynthesis of growth-stimulating materials occurred in the living, damaged cells at a rate comparable with the loss of such materials to the intercellular fluids. Similar evidence indicated the synthesis of substances having ultra-violet absorption characteristic of nucleotides, but at a rate greater than that of their diffusion into the suspension medium.

The above observations indicate that the mechanism of the release of proliferation-promoting factors by slowly-injured cells involves an increased membrane permeability arising as a result of injury, accompanied by a diffusion of various non-protein substances into the intercellular fluids and a resynthesis of certain of these materials within the living, injured cell as their concentration is disturbed by diffusion.

I wish to acknowledge the kind assistance of Mrs Rosaline K. Abramowitz in carrying out the growth tests, of Mr Roy Slaunwhite in preparing the monochromatically-irradiated materials, of Mr Robert Sinsheimer for assistance in the experiments with autolysates, and of Dr Dorothea G. Loofbourow for assistance in obtaining the absorption spectra of various preparations.

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73. A Necessary Precaution in the Use of Takadiastase for the Estimation of Maltose

By **Arthur Hugh Bunting**, *From the Department of Botany, Oxford*

(*Received 13 July 1942*)

Of recent years enzymic, rather than purely chemical, methods of hydrolysis have become increasingly popular in biochemical analysis, especially in that of carbohydrates and carbohydrate mixtures. It is customary to add toluene to the enzyme-substrate mixture in the hope of ensuring sterility during the period of incubation. During some investigations involving considerable numbers of incubations the writer became doubtful of the efficiency of toluene in this respect, and it was necessary in the case of the maltose-glucose hydrolysis to investigate the position more critically.

Yemm [1935] used takadiastase to hydrolyse maltose in the analysis of sugar mixtures derived from barley seedlings. He assumed that maltose, being composed of two glucose units but possessing only one reducing group, should on hydrolysis show an increase in reducing power of 100%. His experiments, in which toluene was used as an antiseptic, gave him an increase of rather less than 100% as measured by the Hagedorn-Jensen technique. The observed increases lay in fact between 90 and 97% of the original value. This, he suggested, might be due to the establishment of a glucose-maltose equilibrium. This explanation involves the supposition that maltose was being synthesized to a significant extent during incubation. While such syntheses have been demonstrated, it is unlikely that in the dilute solutions now under consideration the extent of this synthesis would markedly affect the position of the equilibrium point. Yemm allowed for the discrepancy between his observed and expected results by assuming a conventional increase of 95% and calculating accordingly. In view of the small amounts of maltose he was measuring in relation to the quantities of other sugars present, it is in the highest degree unlikely that his assumption involved any significant degree of error in his conclusions.

The present writer was induced to examine the position more closely by finding the same degree of discrepancy between the expected and observed increases in the reducing power of maltose solutions after takadiastase hydrolysis.

In the first place, the assumption that the 'theoretical' increase in reducing power on hydrolysis is 100% is unsound. This question was investigated using the Shaffer & Somogyi [1933] method of measuring reducing power as modified by Hanes (personal communication). Reagent 60, which gives more complete oxidation of slowly reducing sugars such as maltose, was used.

Dr Hanes, in the same communication, provided a very complete set of figures for the reducing power of maltose solutions with reagent 60; these are reproduced here with his permission in Table 1. Using the same reagent, the writer has obtained figures for the reducing power of glucose (B.D.H. Analar glucose dried for 24 hr. at 80°). These figures are given in Table 2. Curves representing these sets of figures were constructed and used, by interpolation where necessary, for the calculations which follow.

The reducing power of 1 mg. maltose is equivalent to 1·75 ml. $N/100$ $Na_2S_2O_3$. 1 mg. maltose will yield 1·053 mg. glucose on hydrolysis. The reducing power of 1·053 mg. glucose is 3·65 ml. $N/100$ $Na_2S_2O_3$. This is an increase of 1·90 ml. or 109%. This is therefore the 'theoretical' expectation of the increase in the reducing power of maltose after complete hydrolysis.

Table 1. *Reducing power of maltose solutions with Shaffer-Somogyi reagent 60* (Data of C. S. Hanes)

Maltose mg./5 ml.	$Na_2S_2O_3$ ml. $N/100$	Maltose mg./5 ml.	$Na_2S_2O_3$ ml. $N/100$	Maltose mg./5 ml.	$Na_2S_2O_3$ ml. $N/100$	Maltose mg./5 ml.	$Na_2S_2O_3$ ml. $N/100$
0·1	0·17	0·5	0·85	1·4	2·50	3·0	5·56
0·2	0·34	0·6	1·03	1·8	3·26	3·5	6·55
0·3	0·51	0·8	1·385	2·2	4·03	4·0	7·57
0·4	0·68	1·0	1·75	2·6	4·80	—	—

Table 2. *Reducing power of glucose solutions with Shaffer-Somogyi reagent 60*

Glucose mg./5 ml.	$Na_2S_2O_3$ ml. $N/100$	Glucose mg./5 ml.	$Na_2S_2O_3$ ml. $N/100$	Glucose mg./5 ml.	$Na_2S_2O_3$ ml. $N/100$	Glucose mg./5 ml.	$Na_2S_2O_3$ ml. $N/100$
0·5	1·65	1·0	3·42	1·5	5·24	2·0	7·11

The most obvious explanation of the very considerable discrepancy between 109% and ~ 95% thus revealed was bacterial contamination. The maltose and takadiastase preparations were therefore cultured on agar plates. No micro-organisms grew from the maltose, but the takadiastase preparation was found to be heavily infected. Counts of the plates indicated that there were approximately two million organisms per g. of powder.

By the use of a 'Technico' filter with Seitz E.K. bacterial filter pads and the appropriate aseptic technique, a 1% solution of takadiastase was prepared which was shown to be sterile. It produced the maximum increase in the reducing power of maltose solutions in 42 hr. at 30° under aseptic conditions. The theoretical conclusion reached above was now examined in practice. A solution of a B.D.H. preparation of maltose had a reducing power, on appropriate dilution, of 3·25 ml. $N/100$ $Na_2S_2O_3$ per 5 ml. This is equivalent to 1·80 mg. maltose according to Hanes's figures. On incubating part of the original solution with takadiastase under sterile conditions the reducing power, again after dilution and after subtraction of the takadiastase blank value, was 6·82 ml. $N/100$ $Na_2S_2O_3$, an increase of 110%. This corresponds to 1·92 mg. glucose; the calculated amount is 1·90 mg.

This indicates that micro-organisms were responsible for the low values found by Yemm and, initially, by the writer. It seems clear that the uncritical use of toluene in work of this kind may give rise to considerable errors which will not be constant in magnitude. It is advisable, if an aseptic technique cannot be employed, to use a more effective antiseptic such as thymol. The writer has found that this substance, if added in sufficient quantity, will ensure sterility in plant preparations and extracts, over considerable periods of incubation. The proportion of 3 parts by weight of thymol to 1000 parts of fresh material has been found, in comparative trials, to be satisfactory with preparations from seedling barley, in which biochemical reactions were being studied.

The writer is indebted to Dr C. S. Hanes for permission to quote his figures for the reducing power of maltose. His best thanks are also gratefully offered to Dr W. O. James, under whose direction and in whose laboratory the investigations were conducted.

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74. The Catalytic Oxidation of Ascorbic Acid in the Presence of Tea Infusions

By **G. A. Snow** and **S. S. Zilva***, *From the Division of Nutrition, Lister Institute, London*

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The experiments described in this communication originated in an observation made in connexion with a wider inquiry into the suitability of certain articles of food as carriers of ascorbic acid. It was found that condensed milk to which synthetic ascorbic acid was added, lost its antiscorbutic activity when mixed with tea. In an effort to prevent this destruction, in a way that could conveniently be applied in practice, information of theoretical interest was obtained which may have a bearing on other problems. It was established that the inactivation of the ascorbic acid was due to the presence of certain organic compounds in the infusions which were capable of catalysing the oxidation of ascorbic acid in the presence of O_2. For the sake of convenience these catalysts will be referred to in this communication as C.P. (catalytic principles).

Preliminary observations

In this section some typical experiments are described which illustrate the destructive action of tea infusions on ascorbic acid. It was found that this deleterious effect was observed not only at 100° but also at room temperature. The early experiments were carried out in the presence of milk.

The experimental tea mixture was prepared in a series of evaporating basins each containing:

Tea	1 g.
Unsweetened proprietary condensed milk ...	4 ml.
Ascorbic acid	7 mg.
Tap water	96 ml.

The milk was diluted with part of the water and the ascorbic acid was dissolved in the diluted milk. Each solution was made up to volume and poured on the tea in the basin and mixed. The mixtures were then heated so as to bring them to the boil in about 15 min. and then kept at a temperature of about 80° for 30 min. before cooling. The loss of ascorbic acid was ascertained immediately the mixtures reached the boiling point and also at various intervals subsequently and corrections were made for diminution in volume due to evaporation. In experiments carried out at room temperature the ascorbic acid was added after the infusion had cooled. For the assessment of the ascorbic acid the mixtures were precipitated with an equal volume of 5% trichloroacetic acid. After centrifuging, an aliquot quantity from the middle layer between the fat and the coagulated protein was removed and titrated with 2:6-dichlorophenolindophenol.

It is seen from Table 1 that on reaching the boiling point about 50% of the ascorbic acid had disappeared and this disappearance continued, so that after 90 min. (30 min. at 80° and 60 min. cooling), the destruction was almost complete. A small amount of the ascorbic acid which had disappeared could be recovered as dehydroascorbic acid. At room temperature the disappearance of the ascorbic acid took place much more slowly. Even in this case the loss of the vitamin in its reduced form after 150 min. amounted to about a half. As might have been expected, more dehydroascorbic acid was found when

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Table 1. *Loss of ascorbic acid in the presence of tea infusions containing milk*

1. Infusions brought to the boil and then maintained at approx. 80°.

	% loss of added ascorbic acid									
	Before treatment with H_2S					After treatment with H_2S				
Time min. ...	0	10	20	30	90	0	10	20	30	90
Exp. 1	33	32	46	50	—	15	16	24	38	—
2	51	69	74	81	93	46	70	64	75	92
3	59	67	—	83	88	37	56	56	69	66

(The times were measured from the point when the infusions reached 100°.)

2. Infusions kept at room temperature.

	% loss of added ascorbic acid								
	Before treatment with H_2S				After treatment with H_2S				Control, tea absent
Time min. ...	15	30	60	150	15	30	60	150	150
Exp. 1	19	22	32	44	—	—	—	—	3
2	13	23	31	45	0	18	—	26	3
3	9	20	33	51	5	7	10	20	2
4	11	21	34	50	0	0	2	13	4

the destruction took place at room temperature than at the higher temperatures. Only half of the destroyed ascorbic acid was irreversibly lost, the remainder being converted to the more labile, but physiologically active, dehydroascorbic acid.

Technique

In order to study this phenomenon with greater precision a more elaborate technique than the one employed in the preliminary work had to be adopted. Buffered tea infusions not containing milk were used.

The C.P. activity was determined by measuring the initial rate of oxidation of ascorbic acid at *p*H 7 at a temperature of 38°. The reaction was carried out in 50 ml. conical flasks supported in a thermostatically controlled water bath and shaken at the rate of 110 complete oscillations per minute which ensured the maximum rate of oxidation. The constitution of the reaction mixtures was as follows:

5 ml. aqueous solution containing the C.P. preparation and other added substances.

4 ml. phosphate buffer containing $10^{-3}M$ KCN.

1 ml. 0·4% solution of ascorbic acid in buffer.

The ascorbic acid solution was added after the remainder of the mixture had reached the temperature of the water bath. After a further 30 sec. the initial ascorbic acid concentration was determined in 0·5 ml. of the mixture. This was repeated six times at equal intervals ranging from 1 to 10 min. according to the activity of the C.P. The titrations were carried out in small glass thimbles of about 3 ml. capacity. The burette was so constructed that the indicator was delivered through a capillary which dipped into the solution in the thimble and which was oscillated by an electromagnetic device, thus ensuring constant and thorough stirring. Under these conditions the error did not generally exceed ±0·5%. The most important precaution in these determinations was the control of *p*H, since, as will be seen later, very small deviations can cause significant changes in the rate of oxidation. Care was therefore taken to ensure that every solution in the experiments was at *p*H 7·0 and that the resulting reaction mixture was exactly neutral. When this was not possible the *p*H was determined electrometrically and corrections were made accordingly. For greater accuracy it was also desirable that in assessing the initial rate of the oxidation, measurements should be taken during the early stages of the reaction, i.e. during the oxidation of about a quarter of the total ascorbic acid present, when the rate of the reaction was only slowly diminishing.

The destruction of ascorbic acid was followed up by subtracting the subsequent titrations from the initial titrations. The obvious way of determining the initial velocity was by plotting these differences against time and drawing a smooth curve through the points. In doing this it was found that the curvature of such graphs was never great and that frequently they were almost rectilinear. The slope of the tangent at the origin was determined by the use of a tangentimeter similar to that described by Simons [1941], and from this slope the initial velocity in μg. of ascorbic acid oxidized per min. was obtained. The form of the curves suggested that a convenient formula for calculation might be derived. Mr S. C. Pearce, of the East Malling Research Station, kindly considered the problem for us. We should like to express here our gratitude to him for the help he has given us. Mr Pearce writes: 'The relationship between the time from the beginning of the reaction, t, and the quantity of ascorbic acid present at that time, Ω_t, is approximately rectilinear, and it was decided to investigate the fit given by the simplest generalization of this relationship, namely by the second degree law

$$\Omega_t = a + bt + ct^2.$$

'Each trial yielded seven observations, ω_0, ω_1, ω_2, ... ω_6, at times 0, τ, 2τ, ... 6τ respectively; the parameters, a, b, and c were chosen so as to make as small as possible the squared deviations between the observed and predicted results, i.e. so as to minimize $\sum_{n=0}^{6} (\omega_n - \Omega_{n\tau})^2$. That this procedure is reasonable is shown by the theorem of Markoff [cf. David & Neyman, 1938], while the assumption that all observations are equally liable to error is probably justified. The curve so fitted gives the initial velocity of the reaction as

$$\frac{13\omega_0 + 2\omega_1 - 5\omega_2 - 8\omega_3 - 7\omega_4 - 2\omega_5 + 7\omega_6}{28\tau}$$

and the quantity of ascorbic acid initially present as best estimated by

$$\frac{32\omega_0 + 15\omega_1 + 3\omega_2 - 4\omega_3 - 6\omega_4 - 3\omega_5 + 5\omega_6}{42}.$$

In a series of 50 experiments the results obtained by both methods were compared and good agreement was found to exist between the two. The use of the formula was eventually adopted because it avoided subjective errors involved in drawing the curves and in estimating the slope of the tangents. The figures for reaction velocities given in the tables always refer to the loss of ascorbic acid from 10 ml. (the initial volume) of the reaction mixture. All the data given in this communication are those of representative experiments.

Catalytic nature of the oxidation

It was desirable to ascertain whether the oxidation of ascorbic acid in the presence of tea infusions was due to a non-catalytic chemical oxidation by some substance present in tea such as a quinone, or whether the reaction was due to the presence in it of substances which catalysed an oxidation process in which molecular O_2 was involved. The experiments to be described in this section leave little doubt that the latter assumption is correct.

If the substances responsible for the oxidation of ascorbic acid were acting as a catalyst no stoichiometric relationship should have existed between it and the vitamin and the oxidation should have proceeded for long periods of time. This was actually found to be the case, as will be seen from Table 2. Allowing for the loss in the control vessel, we see that 59 mg. ascorbic acid were oxidized through the agency of 20 ml. of an infusion, prepared by extracting 0·2 g. tea with 100 ml. water, which contained 15·5 mg. of dry matter, only a part of which could have consisted of the C.P. This observation is not in consonance with a non-catalytic reaction.

Table 2. *The disappearance of large quantities of ascorbic acid in the presence of tea infusion at room temperature*

Time days	mg. ascorbic acid lost: Tea infusion present	Control	Time days	mg. ascorbic acid lost: Tea infusion present	Control
1	14	2	4	65	16
2	31	7	5	77	18
3	49	11			

The solution contained 20 ml. tea infusion made from 0·2 g. tea extracted with 100 ml. water, 10 ml. phosphate buffer, 100 mg. ascorbic acid dissolved in 10 ml. buffer and $10^{-2}M$ KCN. In the control flask the tea infusion was replaced by water. The ascorbic acid was estimated by titration of 0·5 ml. samples at 24 hr. intervals.

Table 3. *The aerobic nature of C.P. activity*

	Contents of tubes (ml.)					
Ascorbic acid solution	0·5	0·5	0·5			
Tea infusion	2·0	—	—			
Buffer solution	2·5	2·5	2·5			
Benzoquinone solution	—	2·0	—			
Water	—	—	2·0			
	Air absent			Air present		
% ascorbic acid lost after 45 min.	3	93	1	88	96	18

Tea infusion was prepared from 1 g. tea extracted with 100 ml. water. Ascorbic acid solution: 20 mg. in 50 ml. water. Buffer solution: phosphate-citrate, *p*H 6·8. Benzoquinone solution: 0·1% in water.

In another experiment the oxidation of ascorbic acid in the presence of tea infusion or of benzoquinone was followed up in modified Thunberg tubes [Keilin, 1929] under aerobic and anaerobic conditions. In the latter case the tubes were evacuated, washed out three times with N_2 and finally evacuated, after which the ascorbic acid in the stoppers was mixed with the contents in all the tubes. Table 3 gives the details and results of the experiment. It will be noticed that whereas the vitamin was oxidized by the quinone in the absence as well as in the presence of air, in the case of the C.P., oxidation occurred only when O_2 was present. This observation proves that O_2 is utilized in the reaction. It may be mentioned in this connexion that no substance capable of effecting the anaerobic oxidation of ascorbic acid was generated in tea infusions after prolonged shaking in air.

The following experiment, which was devised mainly for the purpose of establishing the relationship between the loss of ascorbic acid through the agency of the C.P. and the absorption of O_2, throws some light on the way the O_2 may be utilized in the reaction. The Warburg-Barcroft apparatus was employed. Each of the flasks contained a mixture of tea infusion and buffer solution in the main vessel and a solution of ascorbic acid in the side bulb. $0{\cdot}5 \times 10^{-3}M$ KCN was added in every case. After the flasks had reached the temperature of the bath (38°) the ascorbic acid in the side bulbs was mixed with the solutions in the main vessels. Individual flasks were removed at intervals, the contents acidified and titrated with indophenol. At the same time the O_2 uptake was recorded in the corresponding flasks in the bath. Table 4 shows that the O_2 absorbed corresponds to the approximate uptake of 1 mol. O_2/mol. ascorbic acid and the reaction appears to proceed thus:

$$\text{Ascorbic acid} + O_2 \rightarrow \text{dehydroascorbic acid} + H_2O_2.$$

That dehydroascorbic acid is formed in the reaction has already been demonstrated by us (cf. Table 1). That H_2O_2 is also produced is suggested by the results given in Table 5, which show that in the presence of catalase the O_2 uptake approximates only $\frac{1}{2}$ mol. instead of 1 mol./mol. ascorbic acid. A more accurate correspondence between the

Table 4. *Relationship between the disappearance of ascorbic acid and the absorption of O_2 in the presence of tea infusion*

Time min.	Loss of ascorbic acid μ mol.	O_2 absorbed μ mol.	Time min.	Loss of ascorbic acid μ mol.	O_2 absorbed μ mol.
5	—	0·29	30	1·51	1·56
10	0·68	0·65	45	1·99	2·07
15	—	0·87	60	2·61	2·52
20	1·14	1·09	75	2·96	2·90
25	—	1·32	90	3·32	3·17

Each flask contained 1·0 ml. tea infusion prepared by extracting 0·2 g. tea with 100 ml. water and 0·5 ml. phosphate buffer *p*H 7·0 in main vessel. 0·5 ml. ascorbic acid solution (16 mg. in 10 ml. buffer) was placed in the side bulbs and mixed with the other solutions at zero time. $0{\cdot}5 \times 10^{-3}$ *M* HCN was present in the solutions.

Table 5. *Relationship between the disappearance of ascorbic acid and the absorption of O_2 in the presence of tea infusion and catalase*

Time min.	Loss of ascorbic acid μ mol.	O_2 absorbed μ mol.	Time min.	Loss of ascorbic acid μ mol.	O_2 absorbed μ mol.
5	—	0·12	45	—	1·19
10	—	0·27	50	2·61	1·31
15	—	0·50	60	3·42	1·49
20	1·59	0·55	70	—	1·65
25	—	0·73	80	3·56	1·79
30	2·00	0·87	95	3·74	2·07
35	—	0·98	120	4·20	2·37
40	2·15	1·13			

Each flask contained 0·5 ml. tea infusion prepared by extracting 0·4 g. tea with 100 ml. water, 0·5 ml. catalase preparation containing 0·16 mg. solid matter, and 0·5 ml. phosphate buffer *p*H 7·0 in main vessel. 0·5 ml. ascorbic acid solution (20 mg. in 10 ml. buffer) was placed in the side bulbs and mixed with the other solutions at zero time. $0{\cdot}5 \times 10^{-3}$ *M* sodium urate was present in the solutions.

figures could not be expected because controls showed that some ascorbic acid disappeared without any uptake of O_2 in the absence of tea infusions. The latter also absorbed O_2 very slowly themselves. It may be pointed out that suitable concentrations of the catalase preparation and the C.P. had to be established by trial, since the enzyme and the C.P. in tea tended to inactivate each other.

The course of the destruction of ascorbic acid by the catalytic principles present in tea infusions

The tea infusions were buffered at *p*H 7·0 in *M*/20 phosphate solution. The course of the reaction in a typical experiment is shown in Fig. 1. The rate of disappearance of ascorbic acid remained fairly uniform during the first part of the reaction but gradually fell off as the concentration of the vitamin decreased. Towards the end, when most of the ascorbic acid had been oxidized, the fall in the reaction velocity became very marked.

Influence of p*H on the catalytic destruction of ascorbic acid*

The range chosen was from *p*H 5·0 to 8·0. Values lower than 5·0 tended to cause precipitation of tannin from the tea infusions, whilst above 8·0 it was impossible to avoid a rapid loss of ascorbic acid even in the absence of tea infusion. The buffer solutions used in these experiments were McIlvaine's [1921] phosphate-citrate mixtures, and the exact *p*H was determined by a glass electrode after addition of the tea infusion. It was found that the initial rate of reaction increased very rapidly with rise in *p*H. Fig. 2 shows that the log of the initial reaction velocity was directly proportional to the *p*H over the range investigated. This fact was later employed to provide a correction for small variations in *p*H in other experiments described below. From the slope of the curve in Fig. 2 it is seen that the log velocity increased by 0·65 for a rise of one *p*H unit.

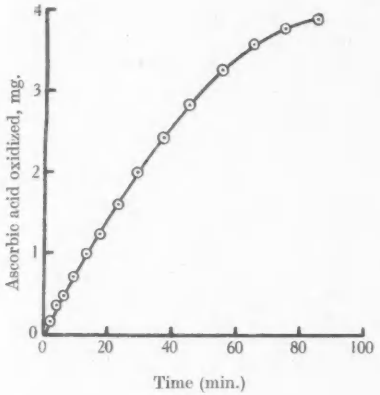


Fig. 1.

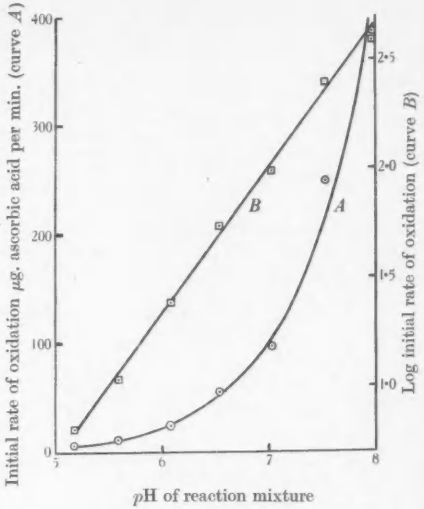


Fig. 2.

Fig. 1. The disappearance of ascorbic acid in the presence of tea infusion. Reaction mixture: 5 ml. tea infusion prepared by extracting 0·2 g. tea with 100 ml. water, 5 ml. phosphate buffer *p*H 7·0, 4 mg. ascorbic acid and 10^{-3} *M* HCN.

Fig. 2. The effect of *p*H on the rate of oxidation of ascorbic acid in the presence of tea infusion. Infusion prepared by extracting 0·2 g. tea with 100 ml. water. Figures are calculated on the basis of 5 ml. infusion.

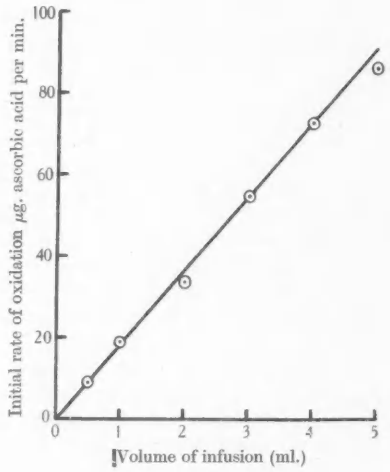


Fig. 3.

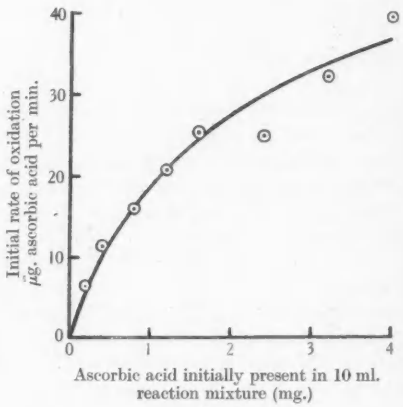


Fig. 4.

Fig. 3. Effect of varying tea concentrations on the rate of oxidation of ascorbic acid. Infusion prepared by extracting 0·3 g. tea with 100 ml. water.

Fig. 4. The rate of oxidation of varying concentrations of ascorbic acid by a tea infusion. Infusion prepared by extracting 0·15 g. tea with 100 ml. water. 2·5 ml. present in each flask.

Influence of tea concentration on the rate of oxidation of ascorbic acid

The rate of oxidation of ascorbic acid was measured in the presence of varying amounts of a tea infusion. A typical result is given in Fig. 3, from which it will be seen that the rate of the reaction was directly proportional to the amount of tea infusion present, a result which was in consonance with the catalytic nature of the reaction.

Influence of ascorbic acid concentration on the rate of oxidation in the presence of tea infusion

The initial rate of oxidation of ascorbic acid in the presence of a constant amount of tea infusion increased with increasing concentrations of the substrate, but not in a directly proportional manner. As will be seen from the experiment represented in Fig. 4 the influence of increasing amounts of ascorbic acid became less marked as the concentration rose, which indicated that it was possible to saturate the C.P. with excess of ascorbic acid.

The influence of various substances upon the C.P. *activity of tea infusions*

It was of interest to compare the catalytic action of tea infusion upon the oxidation of ascorbic acid with that brought about by Cu ions. A number of substances known to inhibit the latter type of oxidation, namely sodium chloride, cyanide, glycine, cysteine, iodide, pyrophosphate, proteins, 8-hydroxyquinoline and uric acid [Giri & Krishnamurthy, 1940], have been investigated, in order to compare their inhibiting effect on the C.P. with their action on Cu. It was necessary to correct the figures for changes in *p*H caused by the addition of these substances, using the method described above. The

Table 6. *Influence of various substances on the* C.P. *activity of tea infusions*

Added substance		*p*H of mixed solutions	Initial rate of oxidation μg. ascorbic acid per min.		
Name	Concentration		Observed	Corrected to *p*H 7·00	% of rate in absence of added substances
None	—	7·00	38·8	38·8	—
Sodium chloride	0·1 *M*	6·84	40·0	50·8	131
Potassium iodide	0·1 *M*	6·90	59·4	69·0	178
Sodium pyrophosphate	0·01 *M*	6·98	36·6	37·8	97
Glycine	0·05 *M*	6·97	30·8	32·2	83
8-Hydroxyquinoline	0·005 *M*	7·12	41·4	34·6	89
Dimedone	0·1%	6·90	24·0	27·8	72
Egg albumin	0·2%	7·03	19·0	18·2	47
Cysteine	0·002 *M*	7·00	24·4	24·4	63

Each flask contained 2·5 ml. tea infusion prepared by extracting 0·2 g. tea with 100 ml. water, 2·5 ml. of an aqueous solution of the added substance adjusted to *p*H 7·0, 5 ml. phosphate buffer *p*H 7·0, 4 mg. ascorbic acid and $0{\cdot}5 \times 10^{-3}$ *M* HCN.

Table 7. *Influence of cyanide and urate on the* C.P. *activity of tea infusions*

Added substance	Concentration $M \times 10^{-4}$	Initial rate of reaction, μg. ascorbic acid per min.	% inhibition due to added substance
None	—	45·2	—
Potassium cyanide	1	30·6	32
,,	3	26·8	41
,,	10	21·6	52
,,	30	14·4	68
,,	100	10·4	77
Sodium urate	5	38·4	15

Each flask contained 2·5 ml. tea infusion prepared by extracting 0·15 g. tea with 100 ml. water, 2·5 ml. of an aqueous solution of the added substance adjusted to *p*H 7·0, 5 ml. phosphate buffer *p*H 7·0 and 4 mg. ascorbic acid.

results are shown in Tables 6 and 7. Sodium pyrophosphate, glycine and 8-hydroxyquinoline (Table 6) and sodium urate (Table 7) caused little alteration in the velocity of oxidation, whilst egg albumin and cysteine somewhat inhibited it. The inhibitory action of the protein may be explained by its precipitating effect on the tannins. Milk also exercised a protective action which was probably due to the presence in it of protein. The retarding action of cysteine can be explained by its reducing action on the dehydroascorbic acid formed in the reaction [cf. Borsook, Davenport, Jeffreys & Warner, 1937]. Dimedone which reacts with quinones had also some retarding action. The accelerating effect of NaCl and KI cannot at present be explained.

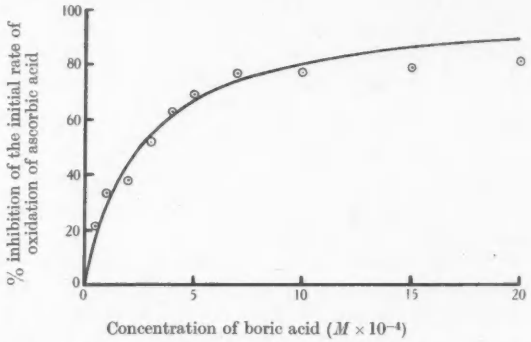


Fig. 5. Inhibition of the C.P. activity of a tea infusion by varying concentrations of boric acid. Infusion prepared by extracting 0·4 g. tea with 100 ml. water. 2·5 ml. present in each flask. The points given represent experimental results. The graph drawn is the hyperbola $y = \frac{100x}{2{\cdot}47 + x}$.

The inhibitory action of cyanide on the Cu-catalysed oxidation of ascorbic acid is very powerful and this reagent was therefore specially studied at various concentrations. Table 7 shows that a cyanide concentration as low as $10^{-4}M$ caused a reduction in the reaction velocity, but that increasing amounts of cyanide did not lead to a proportionate degree of inhibition. Thus, low concentrations of cyanide did not mask the action of the C.P. but probably suppressed other catalytic effects. In view of this observation it was thought advisable to carry out all the experiments in the presence of $0{\cdot}5 \times 10^{-3}M$ cyanide, in order to ensure a higher degree of specificity. Boric acid was found to be a powerful and apparently specific inhibitor of the reaction under investigation. Fig. 5 shows the extent of inhibition produced by varying concentrations of this reagent. It will be seen that, except for the two highest concentrations, the points lie along a hyper-

Table 8. *The inhibiting action of boric acid on the activity of tea infusions of varying concentrations*

Vol. of tea infusion ml.	Initial rate of oxidation, μg. ascorbic acid per min.		% inhibition due to boric acid
	Boric acid absent	Boric acid present	
2·5	103	79·0	23
1·25	51·5*	32·8	36
0·8	33·0*	17·8	46
0·5	20·6*	10·0	51

* Calculated from figure for 2·5 ml. tea infusions.

The tea infusion was prepared by extracting 0·4 g. tea with 100 ml. water. Each flask contained the volume of infusion stated above, together with 5 ml. phosphate buffer *p*H 7·0, 4 mg. ascorbic acid, $0{\cdot}5 \times 10^{-3}M$ HCN and water to bring the total volume to 10 ml. When boric acid was present its concentration was $0{\cdot}5 \times 10^{-4}M$.

bola. It would therefore appear that boric acid formed with the C.P., or with the ascorbic acid, dissociating complexes which had little or no activity. That the complexes were formed with the C.P. is suggested by the results given in Table 8, from which it is seen that the inhibition produced by a given concentration of boric acid became greater as the strength of the tea infusion was diminished. The reaction rates for different concentrations of tea infusion in the absence of boric acid were here calculated from figures obtained at one concentration, by means of the rectilinear relationship which has already been shown to exist between the velocity of oxidation and tea infusion concentration (cf. Fig. 3).

Solubility

The C.P. were found to be insoluble in ethyl acetate, alcohol, acetone, chloroform and benzene. Prolonged extraction with ether removed a considerable amount of matter but comparatively little of the C.P.; the bulk of the active substance, about eight times that present in the ether extract, remained behind. Acetone and alcohol containing 15–50% of water were found to be effective in removing the C.P., but unfortunately these solvents removed at the same time some highly coloured substances from the tea and were therefore less suitable than water in which the C.P. were only sparingly soluble. The conditions for extraction with water were therefore studied in detail. A series of infusions was made with distilled water using varying amounts of tea. The tea and water were mixed, brought to the boil, and boiled vigorously for 10 min. with continual stirring. The leaves were then removed by straining through muslin and squeezed tightly to remove as much liquid as possible. The infusions were cooled, brought to *p*H 7·0 and allowed to stand overnight. All infusions more than 0·6% in strength were found to be turbid and showed a tendency to precipitate on standing. This turbidity disappeared however on the addition of the phosphate buffer in the determination of the C.P. It would appear from Table 9 that the efficiency of the extraction diminished with the increase in the strength of the tea infusions, particularly in those from more than 0·3 g. tea per 100 ml. solution. This point emerges particularly clearly from the ratio of the C.P. activity to the weight of dry matter in the various extracts.

In spite of the sparing solubility of the C.P. in water we found that fairly active solutions could be obtained without heat by extracting ground tea with cold water for several hours with constant stirring.

Table 9. *The efficiency of aqueous extraction of tea at various concentration levels*

Strength of infusion, g. tea extracted with 100 ml. water	Vol. of infusion taken ml.	Initial rate of oxidation μg. ascorbic acid per min.	Wt. of residue from 100 ml. infusion g.	% extraction*		Initial rate of oxidation, μg. ascorbic acid per min. per mg. solid matter
				C.P. activity	Total solids	
0·1	5	55·4	0·0387	—	—	28·6
0·2	5	112	0·0780	101	101	28·7
0·3	3·5	108	0·115	93	99	26·8
0·6	1·8	92·6	0·223	77	96	23·0
1	1·0	54·2	0·366	49	95	14·8
2	1·0	96·8	0·708	44	91	13·7
4	0·8	116	1·22	33	79	11·9
7	0·6	85·6	1·83	18	68	7·8
10	0·5	108	2·48	19	64	8·7

* Figures based on assumption of 100% extraction for 0·1% infusion.

The action of alkali on the C.P. activity of tea infusions

Two tea infusions were used, one prepared from untreated tea and one from tea which had previously been exhaustively extracted with ether; both infusions were adjusted to *p*H 7·0 before use. The effect of alkali treatment under varying conditions is shown in Table 10.

Table 10. *The action of alkali on the* C.P. *activity of tea infusions*

Treatment	Infusion A			Infusion B		
	N NaOH absorbed per 100 ml. infusion ml.	Initial rate of oxidation μg. ascorbic acid per min.	% of reaction rate compared with untreated infusion	*N* NaOH absorbed per 100 ml. infusion ml.	Initial rate of oxidation μg. ascorbic acid per min.	% of reaction rate compared with untreated infusion
None	—	162	—	—	89·2	—
Alkaline solution kept 1 hr. at room temperature	0·07	154	95	0·06	80·3	90
Alkaline solution boiled 1 hr. under reflux in an atmosphere of N_2	0·26	349	215	0·18	137	154
Alkaline solution kept 24 hr. at room temperature in air	0·87	65·6	40	0·31	34·6	39
Alkaline solution kept 24 hr. at room temperature *in vacuo*	0·11	186	115	0·08	127	143
Alkaline solution aerated for 6 hr.	0·62	59·2	37	0·75	55·8	63
Neutral solution aerated for 6 hr.	—	185	114	—	94·6	106

Infusion A = 0·4 g. tea extracted with 100 ml. water. Infusion B = 0·4 g. ether-extracted tea extracted with 100 ml. water. In solutions treated with alkali 2 ml. *N* NaOH were added per 100 ml. infusion. The results are calculated on the basis of 5 ml. of the undiluted infusions.

When the infusions were treated with one-fiftieth of their volume of *N* NaOH, allowed to stand for 1 hr. at room temperature and then brought to *p*H 7·0 by addition of HCl, little change in the C.P. activity was noted. When however the alkaline infusions were maintained at 100° in an atmosphere of N_2 for the same length of time, a marked increase in the C.P. activity resulted and a small amount of the added alkali disappeared. It should be noted that this treatment with NaOH caused a greater increase in C.P. activity with the normal tea infusion than with that prepared from the ether-extracted tea.

The effect of more prolonged exposure of the infusions to alkali at room temperature was also studied. It will be seen that after 24 hr. of the alkali treatment in air there was no increase in activity; on the contrary this treatment led to a marked decrease in activity, at the same time some absorption of NaOH also occurred. When however air was excluded a slight increase in activity was observed, the disappearance of the alkali being only slight. The aeration of an alkaline solution for a shorter period had also an inactivating effect, whilst the passage of air through the infusions at *p*H 7·0 caused little alteration in activity. It would appear that the C.P. are inactivated by oxidation and that this destruction proceeds at an appreciable rate in alkaline solutions exposed to air.

Acetylation and benzoylation of tea preparations

These experiments were carried out in an attempt to isolate the active principles. Dry preparations exhibiting C.P. activity were used in preparing the derivatives. In order to obtain fuller information two different methods of extraction were employed.

Preparation 1. 10 g. tea were boiled for 10 min. with 5 l. water and then filtered and concentrated to 100 ml. at atmospheric pressure. The active solid matter which separated was filtered off, dried, washed with acetone and finally dried *in vacuo*. The yield was 1·15 g., representing approximately 30% of the solid matter extracted.

Preparation 2. 250 g. tea were ground finely, mixed with 1 l. cold water and heated to about 100°. The tea was then thoroughly pressed to remove the absorbed water and dried.

150 g. of the dried product, after extraction with ether in a Soxhlet apparatus for 240 hr. (residue 130 g.), were boiled for 1 hr. with a mixture of 5 l. acetone and 1·5 l. water. After filtration the extract was evaporated to dryness under reduced pressure; it yielded 12·5 g. solid active material.

Acetylation of the solid preparations was accomplished by refluxing 1 g. of the material with 1 g. of fused sodium acetate and 5 ml. of acetic anhydride for 1 hr. The products were separated by pouring the mixture into water and were thoroughly washed and dried. Benzoylation was carried out by the usual Schotten-Baumann method. 1 g. of the tea preparation was dissolved in 10 ml. 2*N* NaOH and treated with 4 ml. benzoyl chloride. The product which separated out was thoroughly washed, dried, dissolved in chloroform and precipitated with light petroleum.

It soon became evident that by these esterifications at least two fractions could be obtained from each derivative, one of which was soluble in benzene or ether and the other insoluble. Both fractions showed activity on hydrolysis. The relative amounts of the two fractions varied according to the method used in preparing the tea extract. None of the acetylated or benzoylated products was appreciably soluble in water or in cold NaOH. Suspensions obtained by diluting acetone solutions with water were found to be entirely devoid of C.P. activity. It was not possible to recrystallize any of the derivatives. Although they were fairly soluble in acetone, chloroform or acetic acid, concentration of the solutions led merely to the precipitation of amorphous material. The above derivatives were not active in themselves but became so on hydrolysis with dilute aqueous NaOH. The fractions insoluble in benzene were fully hydrolysed by 0·1*N* alkali at 100° in 10–15 min., whilst the benzene-soluble fractions required a somewhat longer time with 0·5*N* NaOH. The hydrolysed products were examined for C.P. activity by the usual method. In the case of the acetyl derivatives the acetyl values were at the same time determined by steam distillation. The activity of the preparations was calculated per mg. of the dry product before hydrolysis. In this way it was possible to calculate the degree of recovery of the C.P. in the hydrolysed products by comparison with the original tea preparation treated with NaOH under similar conditions. The figures given in Table 11

Table 11. *Acetyl and benzoyl derivatives of active tea preparations*

Derivative		% benzene-soluble fraction in crude derivative	Acetyl content % CH_3CO	C.P. activity of solutions obtained by alkaline hydrolysis: Initial rate of oxidation μg. ascorbic acid per min. per mg. derivative	C.P. activity of solutions obtained by alkaline hydrolysis: % recovery compared with activity of original preparation
Tea preparation 1		—	—	32·6	100
Acetyl derivative from 1	Benzene-soluble fraction	19	33·4	16·6	76
	Benzene-insoluble fraction	—	34·5	23·8	111
Benzoyl derivative from 1	Benzene-soluble fraction	24	—	6·2	42*
	Benzene-insoluble fraction	—	—	10·8	76*
Tea preparation 2		—	—	51·6	100
Acetyl derivative from 2	Benzene-soluble fraction	52	38·7	32·0	101
	Benzene-insoluble fraction	—	37·2	35·0	108

* Calculated on the assumption that benzoyl derivatives have a similar constitution to that of the corresponding acetyl products.

indicate that the benzene-soluble and benzene-insoluble fractions differed little in their acetyl contents; in general both showed an activity of a similar order to that of the original preparation after allowance had been made for the weight of acetyl present. As benzoyl values could not be conveniently obtained, calculations were made for these

derivatives assuming that they had the same constitution as the acetyl products. On this assumption it would appear that the recovery of the C.P. after benzoylation is not so great as after acetylation.

Attempted isolation of the catalytic principles

The activity was entirely removed by precipitation with an excess of neutral Pb acetate and could be regenerated from the precipitate by treatment with H_2SO_4 in an aqueous acetone solution. Attempts to concentrate the active material by fractional precipitation by successive additions of Pb acetate or by variation of the *p*H were unsuccessful. A partial removal of the C.P. activity by precipitation could also be brought about by saturating the infusions with NaCl, or by the addition of mineral acid or caffeine. The catalytic material was readily absorbed by aluminium hydroxide precipitated in the solution, and less easily by 'active' Al_2O_3 or norit charcoal. Some elution could be effected from the latter absorbent by means of aqueous acetone, but in the case of Al_2O_3 the combination appeared to be chemical rather than physical and it was therefore not found possible to elute the active substance. Equally unsuccessful were attempts at the separation from buffered tea infusions by the chromatographic method using the following absorbents: calcium carbonate, calcium sulphate, kieselguhr and silica. The preparation of *p*-azobenzenebenzoic acid esters and their subsequent separation by chromatography also did not yield satisfactory results in our hands.

As has already been mentioned, the C.P. activity is lost on benzoylation or acetylation, is recovered after hydrolysis of the esters and is slowly lost by aerobic oxidation in alkaline solution. All these properties correspond with those of the polymerized tannins present in manufactured tea and it was therefore reasonable to suppose that the substances responsible for catalysing the oxidation of ascorbic acid were closely associated with the tannin fraction. The inhibitory action of boric acid also suggested that the active substances probably contained several hydroxyl groups. As preliminary attempts had shown that the isolation of the active substances from tea infusions was associated with great difficulties, it was thought worth while to investigate the activity of substances which, in common with the tea tannins, possess several phenolic hydroxyl groups. The following compounds were therefore tested for their catalytic action on the oxidation of ascorbic acid in the presence of cyanide: *d*-catechin, a stereoisomeride of *l*-epicatechin, which is an important constituent of the tannin fraction in fresh tea leaves [Tsujimura, 1929; Deijs, 1939], tannic acid, catechol, quinol, pyrogallol and gallic acid (Table 12).

Table 12. *Oxidation of ascorbic acid in the presence of various polyhydric phenols*

Compound		Initial rate of oxidation of ascorbic acid μg. per min.	Initial rate of oxidation of ascorbic acid μg. per min. per mg. polyhydric phenol
Name	mg. per ml. reaction mixture		
Catechol	5	<2	<0·04
Quinol	5	48·6	0·97
Pyrogallol	5	24·8	0·50
Gallic acid (commercial sample)	2·5	163	6·5
Gallic acid (four times recryst. from water)	1·5	243	16·2
Tannic acid (b.p.)	5	22·6	0·45
d-Catechin	5	<2	<0·04

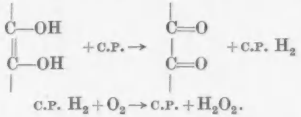
d-Catechin was prepared from cube gambier [Freudenberg & Purrman, 1924]. The tannic acid was a commercial sample presumably consisting mainly of tannins derived from gallic acid; the other reagents were also commercial specimens. Considering that the above compounds were not of the highest purity the very low activity observed may not

have been due to the compounds themselves. Only gallic acid showed any appreciable activity, and even in this case less soluble impurities which became concentrated on repeated crystallization were probably responsible for the catalytic action.

Discussion

There can be little doubt that the aerobic oxidation of ascorbic acid in the presence of tea infusions is catalysed by non-metallic constituents. The oxidation involves the absorption of 1 mol. O_2/mol. ascorbic acid and leads to the production of dehydroascorbic acid and H_2O_2. This could occur according to either of the following two schemes:

1. Ascorbic acid + C.P. → complex
   Complex + O_2 → dehydroascorbic acid + C.P. + H_2O_2.

2. 

   C.P. H_2 + O_2 → C.P. + H_2O_2.

The latter scheme postulates a very low concentration of the C.P. in the tea infusions, since no appreciable oxidation of ascorbic acid occurs in the absence of O_2.

In this investigation all the experiments were carried out in the presence of cyanide, thus excluding the action of metallic, and possibly other, catalysts. The evidence however points to the fact that more than one organic substance capable of catalysing the oxidation was involved in our experiments. It was seen that by extracting the benzoyl and acetyl derivatives with benzene, two fractions were obtained, both of which showed activity on hydrolysis, and also that exhaustive extraction of dry tea leaves with ether removed an active fraction leaving a residue with considerable activity. Although we have not succeeded yet in isolating the active substances the indications are that they are closely associated with, or chemically related to, the tannin fraction of the tea extracts.

The possibility cannot be excluded that substances of a similar catalytic nature may be present in other vegetable and fruit tissues, either originally or after treatments such as cooking, drying etc. In fact, without going into the matter exhaustively, we have already observed that other vegetables exhibit this catalytic activity. Thus aqueous acetone extracts of the root and stem of rhubarb and aqueous infusions of the dried leaves of rhubarb and camelia were found to be active. These may be exceptional sources, but should the presence or formation of such catalytic substances in vegetables and fruits be fairly common it would add one more factor to those which have to be considered in connexion with the stability of vitamin C in vegetables. The problem therefore calls for further inquiry.

Summary

Tea infusions contain constituents capable of catalysing the aerobic oxidation of ascorbic acid in the presence of cyanide. Evidence is produced which suggests that these constituents are closely associated with or chemically related to the tannin fraction of the tea extracts.

A commercial sample of gallic acid showed some catalytic activity whilst *d*-catechin, tannic acid, catechol, quinol and pyrogallol were almost inactive.

The rate of the catalytic reaction has been studied in the presence of varying concentrations of tea and of ascorbic acid and at different *p*H values; it considerably decreases (*ca.* 95 %) between *p*H 7 and 5.

Milk, egg albumin, cysteine and, to some extent, dimedone inhibited the catalytic action, whilst NaCl and KI accelerated it. Sodium pyrophosphate, glycine, 8-hydroxyquinoline and sodium urate did not appreciably influence the velocity of oxidation.

Benzoyl and acetyl derivatives obtained from the extracts, although not themselves active, became so on hydrolysis with alkali at 100°. The alkaline treatment of tea infusions at 100° under anaerobic conditions increased their catalytic activity.

The active constituents of tea infusions were slowly decomposed in alkaline medium by air.

The active constituents formed inactive complexes with boric acid.

The possible mechanisms involved in this catalytic oxidation of ascorbic acid are discussed.

It is pointed out that the presence or formation of catalytic substances of this nature in vegetables and fruits may have to be considered as an additional factor in the stability of vitamin C in vegetables and fruits.

We should like to take this opportunity of expressing our gratitude to Dr R. G. Hatton, Director of East Malling Research Station, for laboratory accommodation. Thanks are also due to Messrs Roche Products Ltd. for a gift of ascorbic acid. One of us (G. A. S.) is indebted to the Medical Research Council for a whole-time grant.

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75. Microdiffusion Methods. Ammonia and Urea Using Buffered Absorbents (Revised Methods for Ranges Greater than 10 μg. N)

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Microdiffusion methods for determinations of minute amounts of ammonia, urea, carbon dioxide, the halogens etc., are now established in many laboratories. A general account of apparatus and several applications have been given elsewhere [Conway, 1939]. Recent extensions include submicro-determinations of total N by Needham & Boell [1939], as well as a further very accurate submicro-total N method by Tompkins & Kirk [1942], also amide- and nitrate-N etc. by Borsook & Dubnoff [1939], quantitative acetone determination by Werch [1940] and an accurate acetone method using bisulphite absorption by Winnick [1941; 1942], who has also extended the technique to lactic acid and threonine estimations.

As shown elsewhere micro-NH_3 determinations by the microdiffusion technique can be brought to any desirable level of accuracy. Recoveries are better than with aeration methods, the apparatus required is simpler and large numbers of determinations can be carried out in one series.

Using the standard unit and 0·2 ml. of the NH_3-containing fluid the full absorption time at room temperature is 45 min., but with rocking this can be reduced to 10 min. Such times arise out of the principles discussed and the experimental results described. (Borsook & Dubnoff [1939] have erred in claiming an absorption time of 90 min. as an advance on the microdiffusion technique.)

The present study is directed especially to the removal of certain objections to the clinical use of the microdiffusion method for urea, but has also a more general application for concentrations of NH_3 beyond a certain level.

The blood urea method as originally described [Conway, 1933] had the disadvantage for clinical use, as commented on by Lee & Widdowson [1937], that the upper limit was 100 mg. urea/100 ml. Occasional blood ureas exceed this figure, though as pointed out [Conway, 1939], the use of 0·1 ml. as well as 0·2 ml. blood in a duplicate estimation raised the limit without any extra trouble to 200 mg./100 ml. However, this disadvantage is fully removed in the method described below, in which there is no upper limit. With this revised method also the amount of cleaning required need not exceed that given to ordinary analytical glassware, nor is any standardization of solutions required, apart from the original making up of $N/50$ HCl, which remains indefinitely stable. This removes the objection raised by Dukes [1939] who considered the microdiffusion method unsuitable for occasional blood urea determinations as distinct from large series.

In the new method for urine and blood urea, as also urine NH_3 or NH_3 in quantities greater than about 10 μg. NH_3-N, there is used the principle introduced into the microdiffusion technique by Abelin [1938], and further studied by Kawerau [1941], of absorbing the NH_3 into a solution such as dilute boric acid and titrating back with standard HCl. Improvements are included arising out of a further study of the conditions of the microdiffusion procedure, an account of which is given at the outset.

The boric acid procedure of Abelin [1938] was criticized by one of us [Conway, 1939] as giving a poor end point with the Tashiro indicator compared with the barium hydroxide titration, but it was noted that the principle was certainly a good one and that a change

in conditions might make it very suitable. This requisite change is described below; it includes the use of an indicator which is more suitable for the purpose, since it changes at an end point where the buffering is minimal and it is more sensitive than the Tashiro indicator when titrating in the alkaline to acid direction; also by a reduction in the strength of the boric acid absorbent (or the use of half-saturated $MgCl_2$) combined with potassium metaborate as alkali. The end point is now quite good and the accuracy of the new blood urea method is practically the same as that of the original, or even superior at the lower levels, while securing in addition some practical advantages.

Before a brief account of the methods, some studies in the microdiffusion conditions are described.

The ideal absorbent for NH_3, with subsequent acidic titration

If NH_3 is absorbed into a boric acid solution in the central chamber of a microdiffusion unit, the *p*H rises rapidly at first and then more slowly as the boric acid buffering comes more fully into play. If, at equilibrium, it rises to a *p*H of 9·4, the NH_3 would be half in the form of undissociated gas, and half as the ammonium cation. The gas would exert the same tension as that in the outer chamber, and if 2 ml. half-saturated potassium carbonate or metaborate were in the outer chamber and 2 ml. boric acid solution in the central, the gas would be shared in the proportion of 1 : 3·4, the relative solubilities being as 3·4 : 1. Hence the proportion of total NH_3 remaining in the outer chamber would be $1/(1+6{\cdot}8)$ or 0·13. If the equilibrium *p*H were less than 9·4 higher proportions would be absorbed and it may be shown that the proportion unabsorbed approximates to

$$U = \frac{bs}{a} \times \frac{10^{-9{\cdot}4}}{10^{-9{\cdot}4}+[\mathrm{H}]},$$

where a and b are the volumes in the inner and outer chambers [H] the equilibrium hydrogen ion content of the absorbent, and s the relative solubility (outer/inner) of the NH_3 gas.

Where b and a are equal and s approximately 0·3, only 0·01 is left (i.e. 99 % is absorbed) at a *p*H of 8·0. Reducing b relative to a and having a greater percentage saturation of potassium carbonate (or better—metaborate) the conditions of absorption are even more favourable. Considering the problem of the buffer strength required to prevent a central chamber rise of over *p*H 8·0, the maximum absorption of NH_3 likely to occur must be known. For blood urea determinations we may consider that 300 mg. urea/100 ml. is rarely exceeded, and if in any case a 99 % absorption is secured at this level, a 97 % efficiency will still be had with 600 mg. urea/100 ml. Using 0·2 ml. blood, 300 mg. urea/100 ml. are equivalent to 1 ml. of $N/50$ NH_3.

Concentrations of buffers with varying *p*K values may be worked out theoretically to give with 2 ml. volumes a *p*H value of 8·0 after absorbing 1 ml. of $N/50$ NH_3. These are given in Table 1, which includes the *p*H of minimal buffering on titrating with standard HCl solution, and the value of this buffering when $N/25$ HCl is used, since such a strength of acid would be suitable for the highest ranges.

It will be seen that theoretically a whole range of buffers almost equally suitable for the purpose could exist, with *p*K values ranging from 11·0 to 8·5, the end point *p*H and the minimal buffering being almost identical. In practice the ideal buffer should also have good keeping power, not affect the indicator used and have the required solubility. Of the many buffers tested only boric acid and $MgCl_2$ were found suitable, boric acid being used in 0·5 % (or in 1 %) strength and $MgCl_2$ as the half-saturated solution (see Table 2). There is little if anything to choose between these two when pure reagents for analysis are used. It may be noted that $MgCl_2$ possesses the added quality—besides the direct buffering power—of reducing the NH_3 tension for a given amount dissolved.

A buffering value of $9{\cdot}5 \times 10^{-5}$ at the end point when titrating with $N/25$ acid (as may be used for very high blood ureas—which are indicated by a special change in the mixed

Table 1

pK of buffer	Conc. of the 2 ml. buffer solution used for absorption	pH of end point giving minimal buffering (after absorption of 0·02 m.eq. NH_3-N)	Buffering (β) at end point $\beta = \frac{d\beta}{dp\mathrm{H}}$ where β is equivalents of acid
11·0	5·0	4·93	$9{\cdot}4 \times 10^{-5}$
10·0	0·50	4·93	$9{\cdot}4 \times 10^{-5}$
9·5	0·16	4·93	$9{\cdot}4 \times 10^{-5}$
9·0	0·055	4·92	$9{\cdot}5 \times 10^{-5}$
8·5	0·021	4·89	$9{\cdot}8 \times 10^{-5}$
8·0	0·010	4·82	$10{\cdot}7 \times 10^{-5}$
7·5	0·0066	4·67	$13{\cdot}6 \times 10^{-5}$
7·0	0·0055	4·47	$20{\cdot}4 \times 10^{-5}$

C (conc. of buffer) $= 0{\cdot}005\,(10^{-8{\cdot}0}/K + 1)$

$[\mathrm{H}]$ (at ideal end point) $= \sqrt{0{\cdot}01\,(10^{-8{\cdot}0} + K) + V \times 1{\cdot}4 \times 10^{-11{\cdot}0}}$

β (buffering at ideal end point) $= 2{\cdot}3\left(\frac{0{\cdot}01 \times 10^{-8{\cdot}0}}{[\mathrm{H}]} + \frac{1{\cdot}4 \times V \times 10^{-11{\cdot}0}}{[\mathrm{H}]} + V \times [\mathrm{H}]\right)$

In the equation for buffering at the ideal end point (or point of minimal buffering) account is taken of the CO_2 of the air and the buffering of the hydrogen ion concentration of the mixture as well as the special buffer used; the buffering of the indicator is not taken into account. End point volume (V) = 2·5 ml.

Table 2

Solution in central chamber	Absorption using 0·2 ml. 0·1 N $(NH_4)_2SO_4$ %	Absorption using 0·4 ml. 0·1 N $(NH_4)_2SO_4$ %
2 ml. ½ sat. $MgCl_2$	98	96
1 ml. sat. $MgCl_2$ adding 1 ml. water before titrating	99	99
1 ml. 0·5% boric acid	93	90
2 ml. 0·5% boric acid	99	98
1 ml. 1·0% boric acid	99	96
1 ml. 2·0% boric acid	100	100

To the 0·2 ml. $(NH_4)_2SO_4$ in the outer chamber 1·0 ml. of saturated potassium metaborate was added to each unit, and the absorption allowed to continue for 90 min. 0·1 N $(NH_4)_2SO_4$ is equivalent to 300 mg. urea/100 ml.

indicator used) means $0{\cdot}2 \times 10^{-3}$ ml. to change the pH by 0·1, a change readily recognized. This corresponds to 0·2 of a small division on the horizontal burette, or to 0·14 mg. urea/100 ml. blood. Titrating with $N/75$ acid as in the usual routine method described, will give the same equivalent value of 0·14 mg. urea/100 ml., or slightly less, as the end point changes. Theoretically therefore the end point should be quite satisfactory, and this will be found so in practice with the indicator described, though when the titration is prolonged, as with very high blood ureas, appreciable CO_2 will be absorbed and the end point when reached will fade a little. On standing a few minutes after such titration a sharp end point will be obtained on adding a little more acid from the burette.

The alkali for use in the outer chamber. As already described [Conway, 1939], the salts of potassium have the most marked action in decreasing the solubility or in raising the tension of NH_3 gas in the outer chamber, and potassium carbonate, metaborate and fluoride were the three most effective salts. Further investigation has shown that though the metaborate and carbonate act almost equally when in half saturated strength in the outer chamber, as the percentage saturation is increased, the metaborate becomes more effective. Fig 1 shows the absorption rates with 1 ml. of saturated potassium metaborate and 1 ml. saturated potassium carbonate added to 0·2 ml. of $(NH_4)_2SO_4$ solution. It will be seen that the metaborate is more effective in equal percentage saturation at the higher levels. The metaborate is likewise more suitable when the solution to be introduced into the outer chamber is acidic, since then no CO_2 will be evolved.

The indicator. In the previous account of the microdiffusion method, Tashiro's indicator was used in the change from an alkaline to an acid direction, and with a maximum sensitivity around a *p*H of 5·4. Here, however, the change is in an alkaline to acid direction with the end point of minimal buffering at *p*H 4·9, hence a combination of bromocresol green and methyl red is more suitable. These are best used in a concentration of $0{\cdot}66 \times 10^{-3}$ % for methyl red and half that strength for bromocresol green. With these may be incorporated phenol red in similar strength to that of the methyl red. This addition, though by no means essential, is an advantage for indicating very high NH_3 values. Thus when NH_3 to the extent of 10^{-2} m.eq. is absorbed the colour of the indicator fades and becomes greyish; with 2×10^{-2} m.eq. absorption it changes to pink and with 4×10^{-2} to a cherry red.

The fixative. For room temperature work, good quality white vaseline has been found satisfactory. When the units are to be incubated the vaseline is hardened by melting with 3 parts of it 1 part of paraffin wax (M.P. 55°). Kawerau [1941] has found liquid paraffin serviceable and his method of applying it may be consulted. Borsook & Dubnoff [1939] used with success glycerol, rendered slightly alkaline, for small microdiffusion units made out of synthetic resin. We have found pure glycerol not as serviceable with the standard unit, as it tends to collect in drops which may fall on to the floor of the unit.

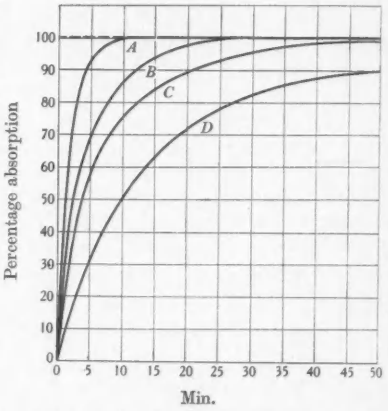

Fig. 1. The curves show the percentage absorption of NH_3 in the standard unit plotted against the time in minutes. *A*, outer chamber contained 0·2 ml. standard $(NH_4)_2SO_4$ and 1 ml. sat. potassium metaborate; rocking maintained at about 60 per min. *B*, same as *A*, but without rocking. *C*, same as *B*, but 1 ml. sat. potassium carbonate used instead of metaborate. *D*, same as *C*, but 1 ml. standard $(NH_4)_2SO_4$ instead of 0·2 ml.

We have found the following water-soluble fixative satisfactory over the period during which it has been examined.

Water-soluble fixative. To 10 parts by weight of powdered gum acacia are added 15 vol. water, 5 vol. glycerol and 5 vol. saturated K_2CO_3. In preparing this fixative, water is added slowly to the powder, the mixture being ground in a large mortar to hasten the solution of the gum. The glycerol and carbonate are then added and stirred in. The mixture may then be used or poured into a large separating funnel and allowed to settle overnight, when the lower fluid can be separated easily from a somewhat frothy upper layer and stored in a stoppered vessel.

For use a few drops are smeared over a glass lid, or, in the manner of Kawerau [1941], some drops are poured on one slide and another is placed on top and drawn across, so that two are prepared together. Results obtained with this fixative have been found to be the same as with vaseline. If traces of NH_3 are present, the mixture may be heated to 100° in a large beaker, with careful stirring throughout to avoid charring, when the NH_3 will be driven off. In the blood urea determinations we did not find any appreciable amount of NH_3 in the fixative.

An alternative and perhaps better water-soluble fixative may be prepared with gum tragacanth, of which much less is required. In preparing this 3 parts of the powdered gum are ground with water which is gradually added until 34 parts are finally included, as well as 15 parts of glycerol and 8 parts of saturated K_2CO_3 (all parts being added by volume except the gum, which is added by weight). The whole forms a soft jelly which may be easily spread on the lids.

Outline of revised NH_3 method for NH_3-N in amounts exceeding 10 μg.

The NH_3-containing fluid in any volume up to 2 ml. is introduced into the outer chamber, but the smaller the volume the more rapid will be the rate of absorption. Into the central chamber is introduced 1 ml. of the boric acid reagent containing indicator (2 ml. are suitable when the NH_3-N exceeds about 100 μg.); alternatively 1 or 2 ml. of half-saturated $MgCl_2$ with indicator may be used. The lid is smeared with the fixative, and after placing in position is slightly displaced in the usual manner to allow the introduction of the alkali (1 ml. of saturated potassium metaborate), then replaced; the outer chamber contents are mixed by rotation and the units left on the bench.

Suitable times for the absorption are as follows:

ml. of NH_3 fluid	No rocking min.	Rocking min.
0·2	45	10
0·5	75	20
1·0	120	60
2·0	240	—

At 38° these times will be reduced to 0·6–0·7 of the above values. At this higher temperature vaseline and hard paraffin 3 : 1 should be used as fixative.

The titration is carried out with standard HCl (*N*/200 or stronger) from the horizontal burette [Conway, 1939] of which an improved type is now available. A 2 ml. Bang burette may also be used, but it will not be found so convenient, especially for a series of titrations.

The end point is sharp, but when the NH_3 content of the analysed fluid is very high and a comparatively long time is taken with the titration, the end point when reached may fade a little. On standing a few minutes, a stable end point is reached by the addition of very little further acid. This fading results from the liberation of some CO_2 absorbed during the titration.

Solutions

Boric acid, 0·5% in 20% alcohol. To make 1 l. of the reagent, 5 g. of the purest boric acid are introduced into a 1 l. flask, 200 ml. alcohol are added, then about 700 ml. distilled water. The boric acid is brought into solution and 10 ml. of the mixed indicator (bromocresol green and methyl red) are added. On mixing, the whole is brought to the desired end point colour of faint reddish, which usually requires the addition of a little alkali, and the mixture made up to the mark.

A further refinement may be secured by adding, after the 10 ml. of mixed indicator, 10 ml. of a phenol red solution (0·066% in alcohol), which serves to indicate very high NH_3 or urea values, when a stronger acid in titrating will be a convenience.

Mixed indicator. This contains bromocresol green, 0·033% and methyl red, 0·066% in alcohol. It keeps indefinitely.

Magnesium chloride. Exactly as for boric acid above, except that 28 g. purest $MgCl_2$ are used instead of the 5 g. boric acid.

Saturated potassium metaborate. 69 g. of the metaborate/100 ml. water are necessary to form a saturated solution at room temperature.

Saturated potassium carbonate. This may also be used instead of saturated metaborate. To prepare a saturated solution 110 g. may be allowed for every 100 ml. water.

Revised method for blood urea

2 hr. method at room temperature. 0·2 ml. blood is delivered into the outer chamber, and 0·5 ml. of urease-phosphate solution is dropped on to it and mixed with it. Into the central chamber are pipetted 2 ml. of the 0·5% boric acid reagent or the $MgCl_2$ solution (this need be only roughly delivered). After 15 min., 1 ml. of saturated metaborate is

added and the outer contents are mixed by rotation. After a further $1\frac{3}{4}$ hr. the lid is detached and the contents of the central chamber are titrated from the horizontal burette with $N/50$ HCl. (For general research purposes where there is no likelihood of very high values $N/100$ may be used.) When very high blood ureas are likely to be encountered it is also a convenience to have a second burette filled with $N/10$ HCl, to be used when the colour in the central chamber is greyish or reddish, if phenol red is included in the absorbing reagent.

If any tendency is experienced to have spills from the central chamber 1·0 ml. of a 1% boric acid reagent may be substituted for the 2 ml.

If a 2 or 3 ml. Bang burette is used, $N/250$ HCl would be a suitable strength, with an alternative burette with $N/100$ for the very high levels. The volume of acid used in ml. is multiplied by 60 to give mg. urea/100 ml. or by 150 when using the $N/100$. Also in using the Bang burette 1 ml. of a 1% boric acid reagent is advisable.

Calculation. The result is obtained by multiplying the number of large divisions (each division = 0·01 ml.) on the burette reading by 3, which gives mg./100 ml. urea in the blood.

A blank may be run at the same time if there is any NH_3 in the reagents, and the burette reading is then subtracted from the previous one before multiplying by 3.

Accuracy of the determination. The end point here is sensitive to about half a small division, or 0·15 mg. urea/100 ml. blood, and when titrating with the horizontal burette the movement of the meniscus should be under perfect control (which is readily secured in the new instrument as supplied by Messrs Gallenkamp). The other errors may be controlled to any desirable level as described elsewhere [Conway, 1939]. From a series of 51 routine duplicate determinations the standard deviation of the individual estimate was calculated as 0·34 mg. urea/100 ml.

Solutions. The boric acid and $MgCl_2$ absorbents are prepared as above, as are also the solutions of metaborate or K_2CO_3.

Urease-phosphate mixture. The glycerol extract of jack bean [Conway, 1939] has been found very satisfactory; it retains its activity for many months in the refrigerator. In the usual routine this was diluted ten times with an equal volume of phosphate solution before use. Kawerau [1941] found that incorporating the phosphate in the original preparation produced an extract which has very good keeping powers and filters quickly. The preparation is made as follows. To 10 g. of jack bean meal 20 ml. of water are added and the mixture is agitated vigorously for about 15 min. (a mechanical stirrer or shaker may be conveniently employed). 100 ml. phosphate buffer at *p*H 7·4 (3 g. anhydrous Na_2HPO_4 and 2 g. anhydrous KH_2PO_4 in 100 ml. water) and 20 ml. glycerol are added. This mixture is shaken well, allowed to stand for a short time and the fluid is decanted on to a large folded filter paper. The mixture filters comparatively quickly and may then be cleared of traces of NH_3 or stored directly. To clear from NH_3 5 g. finely powdered permutit are washed with 2% acetic acid and allowed to settle; the acid is decanted and the permutit washed twice with distilled water. To the permutit is added the urease-phosphate preparation; the mixture is well shaken for some time and allowed to settle, when the clear fluid is decanted off and stored in the refrigerator. A small volume of this is diluted three times when required for a series of determinations.

1 hr. method. 0·5 ml. blood is mixed well with 0·5 ml. of the urease-phosphate mixture (diluted twice) in a small clean dry tube (about 2 ml. capacity) and allowed to stand for 10 min. 0·2 ml. volumes are transferred to the outer chamber of a prepared unit with 2 ml. absorbent in the central chamber, and the unit is closed. 1 ml. of saturated potassium metaborate is then introduced into the outer chamber and the contents are well mixed. After 45 min. the contents of the central chamber are titrated with $N/100$ HCl. The calculation is made as before, the number of large units on the burette being multiplied by 3·0. The accuracy is not appreciably less than in the previous method.

It is obvious that by the use of rocking and increased temperatures the time could be further reduced.

Urea in urine. This is carried out by pipetting 0·2 ml. urine suitably diluted to resemble blood strength [Conway, 1939; p. 279] into the outer chamber of a unit containing 1 ml. boric acid reagent and adding 1 ml. saturated potassium metaborate in the usual manner. Titrate with $N/100$ acid.

NH_3 in urine. 0·2 ml. undiluted urine is transferred to the outer chamber of a unit and 1 ml. boric acid reagent into the central chamber. The NH_3 is liberated by 1 ml. saturated potassium metaborate in the usual manner. After 45 min. the titration may be carried out with the $N/50$ acid. Each large division corresponds to 1·4 mg. NH_3-N/100 ml. urine (about 28 large divisions will be the average for the 24 hr. urine).

Discussion

In the blood urea method described, it will be seen that grounds for some previous objections against the microdiffusion method for clinical use are removed. There is, practically speaking, no upper limit, the titrating fluid is perfectly stable, as is also the absorbing fluid, and the cleaning of the units requires no more attention than that given to analytical glassware, but naturally some fixed routine [Conway, 1939] is still advisable. Defective cleaning of the central chamber in an acid-alkali sense reveals itself at once when the absorbing fluid with indicator is introduced. Since, if present, it will be almost always due to the presence of alkali, it may be corrected by a small addition from the burette used to titrate the units, or alternatively, such a unit may be discarded. Apart from the acid used in titration, the only accurate delivery required is that of the 0·2 ml. blood. Errors of the order of 10% in the introduction of the absorbent, the metaborate (or carbonate) or the urease-phosphate solution (provided that the urease preparation is reasonably free from NH_3), are without significance to the determination. The calculation of the result is very simple.

The essential changes in the revised method for ranges $> 10\ \mu g.$ N have proceeded from Abelin's [1938] use of boric acid as absorbent (also studied by Kawerau [1941]). The reduction in concentration of the boric acid in the absorbent, or the substitution of half-saturated $MgCl_2$, with the use of a new indicator more suitable for this particular titration from the alkaline to the acid side, make the end point much more satisfactory. Also the use of potassium metaborate increases somewhat the speed of the absorption, and it is to be noted that full NH_3 absorption can be secured at room temperature in 45 min. using 0·2 ml. of fluid and 1 ml. of the saturated potassium metaborate, and that—at least using standard $(NH_4)_2SO_4$ solutions—complete absorption, if necessary, can be secured in 10 min. by rocking.

Summary

1. A revised microdiffusion method (using the standard unit) is described for estimating quantities of NH_3 greater than $10\ \mu g.$ NH_3-N, and for determining blood urea. Some minor disadvantages of the older method for clinical use have been removed.

2. Further studies of microdiffusion conditions are described.

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76. The Nitrogen of the Potato

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The value of the potato as a source of dietary nitrogen has been examined by several authors, but the interpretation of their results is made difficult, since in nearly all these experiments the total nitrogen only is recorded. The potato tuber contains besides protein a large variety of low-molecular nitrogenous substances, some of little or no nutritive value, and their relative proportions may vary greatly in different samples. Feeding experiments without chemical analysis of the material used will therefore give little information of a useful character.

The data obtained in different countries have recently been summarized [Lampitt & Goldenberg, 1940]; little is known about the nitrogen distribution of domestic varieties. In this paper methods used to characterize the different nitrogenous fractions are described and data for a number of varieties are presented showing fairly wide variations in total nitrogen and in the relative proportions of the different fractions. The distribution of the various fractions over the different morphological layers has also been investigated.

PART I

Methods

Potatoes were stored at 8–10° and usually worked up within one month after lifting. The tubers were cut into slices and quickly frozen and dried at −15°. Chemical changes affecting the distribution of N are unlikely to occur during the drying process since results obtained by this method agree well with those obtained on press-juice. The dried material was then finely powdered and stored.

Most of the total and also of the protein N can be extracted by water or dilute salt solution (Table 1), although 2% NaCl is more efficacious than water for extraction of coagulable N. Increase of the concentration of electrolyte offers no advantage; 2% NaCl was therefore used to extract the total soluble N. Two successive extractions with this solvent supply a representative sample as shown by the following experiment: 140 g. of the dried powder of the variety Dunbar Rover were shaken with 700 ml. of 2% NaCl and the mixture centrifuged. Total and coagulable N were estimated on aliquots of the supernatant fluid. The extractions were repeated eight times using 280 ml. portions of solvent. The relative proportions of the coagulable N in the first and second extractions were 39·8 and 39·9%, whilst in the third and fourth extracts which contained only small amounts of total N the corresponding figures were 31 and 28% respectively. The definite decrease in the percentage of coagulable N in the later extracts may possibly be due to the fact that prolonged extraction removes fairly insoluble low-molecular substances such as purines and tyrosine which are known to occur in potatoes.

Insoluble protein

It appears from Table 1 that a certain amount of N cannot be extracted by salt solutions, acid or alkali. In later experiments it was found that it could not be rendered soluble by alcohol, ether, alcoholic alkali or urea, neither is its extraction facilitated by more thorough grinding. As will be shown later this insoluble N is almost entirely concentrated in the outer layers of the tuber and is probably therefore not formed by de-

naturation of protein during extraction. The residue obtained after repeated extraction with aqueous solvents contained usually between 0·11 and 0·14% N corresponding to about 8–10% of the total N originally present, and gave positive colour reactions with the

Table 1. *Estimation of soluble nitrogen in the potato*

Solvent	mg. N extracted	% of total	mg. coagulable N
Water	175·8	77	56
2% NaCl	23·35	10·4	12
10% NaCl	5·1	2·2	2·3
10% NaCl (2nd extract)	1·1	0·5	1·0
0·2 *N* Na_2CO_3	3·5	1·5	—
Residue	19·6	8·5	—

The potato powder was successively extracted with the solvents.

Millon, Sakaguchi and Hopkins-Cole reagents, the latter being very weak. No positive biuret reaction could be obtained. Digestion with pepsin at *p*H 1·5 rendered about half of the residual N soluble. It may therefore be assumed that at least a large part of the insoluble material is protein. The presence of other substances of unknown chemical character forming part of the cuticular structure cannot however be excluded and the inclusion of the insoluble N in the total protein fraction which is adopted here for the sake of convenience is admittedly somewhat unsatisfactory.

Total protein

The total protein can be determined in two ways: (*a*) the sample is exhaustively extracted and the protein-N in the extracts and residue is estimated; (*b*) a sample is extracted with hot water and N is estimated in an aliquot of the solution after protein has been removed. The second method which is much less laborious gives usually slightly higher results. We have not been able to explain this discrepancy. The values recorded in Table 4 were obtained by the second method.

Estimation of soluble protein (Table 2). The amount of N coagulated by heating at 100° for 1 min. in 2% NaCl and at the slightly acid reaction of the extract is taken as

Table 2. *Comparison of different methods of estimating protein in the aqueous extract*

Precipitant	% of soluble N precipitated	Precipitant	% of soluble N precipitated
Trichloroacetic acid at 0°	40·4	Barium hydroxide and 45% ethyl alcohol	36·9
Trichloroacetic acid at 37°	37·8		
Heating at 80 for 5 min.	37·2	20% lead acetate	42·7
50% ethyl alcohol	—	Copper hydroxide	43·1

measure of the soluble protein-N. Typical protein precipitants remove a similar proportion of the total soluble N; the filtrate from the heat coagulation does not give a precipitate with trichloroacetic acid at 0°. Lead acetate precipitates more N than the other reagents, probably because it forms insoluble complexes with low-molecular nitrogenous substances present in the extract, and the same applies to cupric hydroxide. The small amount of N precipitated by 50% alcohol is noteworthy. A sample of the extract was also filtered through membranes standardized and prepared by Elford's method and put at our disposal through the courtesy of Dr K. Smith. A membrane of the average pore size of 7 mμ retained 41·5% of the total N, whereas one of porosity 13 mμ retained only 35·1%. The filtrate from the first experiment gave no precipitate with trichloroacetic acid, whilst that from the second experiment produced a slight but definite cloudiness. It can be concluded therefore that the same nitrogenous material is precipitated by heat and by trichloroacetic acid and is retained by the 7 mμ membrane; this material is called soluble protein.

Non-protein nitrogen

The total non-protein N was obtained by direct estimation after removal of the protein by heat coagulation. Total amides were estimated by measuring in the Parnas apparatus the ammonia formed on hydrolysis by *N* HCl for 3 hr. at 100°.

Glutamine. Glutamine was estimated according to Vickery, Pucher, Clark, Chibnall & Westall [1935]. This method, which is based on the liberation of ammonia from glutamine at neutral reaction at 100°, is probably not quite specific, since, as pointed out by the authors themselves, other substances such as urea and allantoin may also split off ammonia under these conditions. The method of Pucher & Vickery [1940] which appeared to be very specific did not yield satisfactory results in our hands. A very convenient and in our opinion fairly specific method for the estimation of glutamine was then developed; it is based on experiments, summarized in Table 3. α-Amino acids react quantitatively with ninhydrin to give 1 mol. of CO_2 [Van Slyke & Dillon, 1938]. Aspartic acid is an exception in yielding 2 mol. of CO_2, but in asparagine this anomaly disappears. Glutamine also gives nearly 100% of the theoretical value, i.e. reaction with ninhydrin is very much faster than cyclization. Pyrrolidone carboxylic acid, as might be expected, does not yield any CO_2. If glutamine is heated first for 2–3 hr. at *p*H 6·8 the reaction with ninhydrin disappears nearly completely, whilst asparagine is unaffected; the small amount of CO_2 found consistently in heated glutamine solutions may be due to the formation of glutamic acid by a side reaction.

Glutamine can therefore be estimated by determining the CO_2 developed by ninhydrin treatment both before and after heating at *p*H 6·8. Interference by unstable peptides which might be split under these conditions is a remote possibility which cannot be entirely excluded. Another possible source of error might be cyclization of glutamic acid leading to a loss of reactive carboxyl groups; it appears, however, from the results of Wilson & Cannan [1937] that the rate of such a reaction at neutrality is too low to cause any appreciable error.

Table 3. *Reaction of ninhydrin with amides*

	α-Amino-nitrogen			α-Amino-nitrogen	
	Found %	Calculated %		Found %	Calculated %
Glutamine before heating	9·2	9·55	Asparagine before heating	9·15	9·27
Glutamine after heating at *p*H 6·8	0·37	0	Asparagine after heating	9·25	9·27
Pyrrolidone carboxylic acid	0	0			

The new method was compared with that of Vickery *et al.* [1935*a*] on a sample of potato juice freed from protein by heat coagulation. The ammonia method gave a value of 0·142 of glutamine amide-N per ml., whilst the ninhydrin method gave 0·138 mg. The agreement between these two methods together with the isolation of glutamine, although in poor yield, makes it appear certain that both methods give an accurate estimate of the glutamine content of the potato.

Asparagine. This compound has been estimated as the difference between total amide and glutamine amide. Such a method is somewhat unsatisfactory and it was attempted therefore to utilize the different behaviours of asparagine and aspartic acid with ninhydrin for estimation of the amide. It was assumed that aspartic acid would react with ninhydrin to form in the first place malonic semialdehyde which would then decompose into acetaldehyde and CO_2; in fact no volatile aldehyde could be detected, although Virtanen & Laine [1938] found that a small amount of acetaldehyde was formed in this reaction.

It can actually be assumed that the Sachsse hydrolysis in potato extracts gives approximately correct results for the following reasons. As will be shown later, asparagine has

been isolated in yields up to 80% of that to be expected. Furthermore, other substances which are known to yield ammonia on acid hydrolysis such as urea, ureides and allantoin were shown to be absent or to be present in such small amounts as not to affect the accuracy of the estimation. The most serious objection to this method of estimation is the possibility that amides present in peptide linkage would be estimated as asparagine.

Amino-acid fraction. The total carboxyl carbon or its equivalent of α-amino-N obtained by the ninhydrin method derives from amides and other amino-acids. The difference between total α-amino-N and total amide-N is termed for convenience α-amino-acid-N. The estimations were carried out in the earlier experiments according to Van Slyke & Dillon [1938]; in the later experiments the improved technique of Van Slyke, Dillon, MacFadyen & Hamilton [1941] was used.

Free amino-acids in plant extracts have hitherto been estimated usually by the nitrous acid method of Van Slyke. Experiments were therefore carried out on four samples to compare results obtained by the nitrous acid and the ninhydrin methods. It was shown that the nitrous acid method gave results which were about 15–25% too high, even if allowance were made for the excess N evolved by glutamine [Chibnall & Westall, 1932]. It may be inferred that substances other than α-amino-acids containing amino groups which can react with nitrous acid were present. This error becomes of considerable magnitude if amino-acids are determined in the presence of large amounts of amides and are estimated by difference. Apparent values for amino-acid-N might be obtained which are too high by more than 100%.

Basic N. The N precipitated by phosphotungstic acid at *p*H 1 after keeping overnight at 0° is termed basic N. It is well known that phosphotungstic acid may precipitate non-basic amino-acids and the values for basic N may therefore be too high. Moreover, basic amino-acids are partly estimated both under α-amino-acid-N and under basic N and these two facts are probably the reasons why the fractions add up in most samples to about 104%.

PART II

Isolation of compounds

The most comprehensive work on low-molecular nitrogenous substances of the potato tuber was done by Schulze and his collaborators more than 50 years ago. Very little work involving actual isolation has been done since, the only notable exception being the isolation of glutathione from sprouting potatoes by Guthrie [1932]. Schulze & Barbieri [1877] showed that asparagine accounted for most of the amide; no glutamine could be found, nor could glutamic acid be isolated after acid hydrolysis. In later papers [Schulze & Eugster, 1882; and Schulze, 1904] the presence of amino-acids was demonstrated; arginine was isolated in moderately large amounts, whilst lysine and histidine could be obtained in traces only; tyrosine and leucine were also isolated. Schulze [1883; 1904] also found the bases hypoxanthine, choline and possibly trigonelline in potato extracts. Our isolations can add little to the classical work of Schulze but our justification for recording our results is that modern methods have enabled us to obtain higher yields of substances already found by Schulze and to isolate a few other compounds for which no good methods were available in Schulze's time.

The isolation of asparagine by the method of Vickery, Pucher & Clark [1935] presented no difficulties. Glutamine on the other hand was difficult to obtain although its presence was indicated in varying amounts by the indirect methods discussed above; Steward & Preston [1940] had also obtained evidence that an unstable amide was associated with protein synthesis in potato discs. It seemed therefore quite important to ascertain whether this substance is in fact glutamine. It was found eventually that glutamine

could be isolated from potato extracts if its concentration were at least equal to that of asparagine; if asparagine were in great excess, mercuric nitrate precipitated most of the asparagine and some amino-acids, but practically no glutamine.

The concentration of free amino-acids other than amides in potato extracts is small and their isolation is considerably more difficult than from a protein hydrolysate. No attempt at quantitative isolation was therefore made and only a fraction of the total amino-acids has been accounted for. Tyrosine has been isolated in a yield slightly higher than that obtained by Isherwood [1937; 1938]. Arginine was found in fairly large amounts, whilst lysine and histidine seem to be absent. Qualitative tests for cystine (nitroprusside after reduction and labile sulphur) and for tryptophan were negative on concentrated potato extracts freed from protein. Leucine and phenylalanine, which are known to occur in plant extracts, have been isolated. Volatile bases are absent apart from minute traces of ammonia which were presumably formed by decomposition of glutamine. We have been unable to obtain any hypoxanthine in the purine fraction, but found instead adenine. The phosphotungstic acid precipitate yielded trigonelline and choline. A more convenient and more quantitative method for estimation of choline was precipitation by Reinecke acid. All basic substances give reineckates which are insoluble at acid, or sometimes even at neutral or mildly alkaline reaction, but reineckates of amino-acids, of primary, secondary and tertiary amines and of betaines dissolve at *p*H 12–13 [cf. also Strack & Schwaneberg, 1937]; only quaternary bases without a carboxyl group such as choline and methyl pyridinium chloride give reineckates which are insoluble at such an alkaline reaction. The explanation presumably is that the cationic form of a base forms an insoluble reineckate and quaternary ammonium compounds are so strongly basic that they are ionized even at *p*H 13 and cannot be rendered soluble, as in the case of betaines, by the possession of a carboxyl group which can form a salt. Choline was therefore precipitated with reinecke acid at *p*H 13 and the salt was dissolved in aqueous acetone. This method is probably not quite specific since other quaternary bases such as tetramethyl ammonium compounds may also be precipitated. The fact however that on decomposition of the reineckate choline chloroplatinate is obtained in good yield indicates that the bulk of the reineckate is the choline derivative.

Experimental

Preparation of asparagine. 1·5 kg. of potatoes (Majestic) were ground in a meat chopper and the pulp was mixed with sand and pressed in a hydraulic press which was kindly put at our disposal by Prof. D. Keilin. The residue was again pressed and washed twice with water. The extract containing 1·5 g. of total N and 0·28 g. of asparagine amide-N was then treated with basic lead acetate. The lead precipitate was washed repeatedly on the centrifuge; 400 ml. of the mercuric nitrate reagent [Vickery *et al.* 1935*b*] were then added to the supernatant and the precipitate which formed was decomposed. The yield of asparagine was 2·4 g. which is about 80% of the quantity found by indirect analysis. The recrystallized sample had M.P. 182° (uncorr.). (Found: N, 18·5; amide-N, 9·3; water of crystallization 12·1%; asparagine requires N, 18·7; amide 9·35; water of crystallization 12·0%.) No glutamine could be isolated from the mother liquors, although indirect analysis indicated the presence of 1·2 g. of glutamine in the press-juice. In a later experiment on King Edward potatoes an appreciable quantity of tyrosine was obtained from the mercury precipitate.

Preparation of glutamine. 1000 ml. of press-juice of the medullary parts of King Edward potatoes, which are rich in glutamine, were treated with lead acetate and mercuric nitrate as described above. Asparagine and glutamine were separated by fractional crystallization. Unfortunately, owing to an accident about half of the glutamine solution was lost; 90 mg. of glutamine were, however, obtained which had the following properties: M.P. 182° (uncorr.). (N found 18·65; amide 9·4%; glutamine requires N, 19·2; amide 9·6%.)

Preparation of amino-acids. 5000 ml. of potato extract were precipitated with lead acetate, acetic acid being added to keep the *p*H near 4. The precipitate was then decomposed with H_2S, the H_2S was removed and the solution again precipitated with lead acetate. The combined solutions were concentrated to about 1000 ml. to remove most of the acetic acid, made up to 2000 ml. and adjusted to *p*H 9. The precipitate formed was decomposed with H_2S after addition of HCl; the resulting solution after concentration and adjustment to *p*H 5 deposited 535 mg. of tyrosine. (Found: N, 7·6%; $C_9H_{11}O_3N$ requires N, 7·73%.) Colorimetric analysis by Lugg's [1937] method gave a value of 0·0198% for the original extract which corresponds to 980 mg. in the whole sample; the recovery of crystalline tyrosine was therefore about 55%. The alkaline mother liquor from the tyrosine precipitation was then acidified, excess lead was removed and the solution was again concentrated. Successive crops of crystalline material were obtained which gave after several recrystallizations altogether 10·5 g. of asparagine. The mother liquors from the asparagine crystallizations were combined with the main solution which was now treated with mercuric acetate, sodium carbonate and alcohol [Neuberg & Kerb, 1912]. This precipitate gave after decomposition some more asparagine and a few mg. of tyrosine. The solution after saturation with sodium chloride [Hill & Robson, 1934] yielded a mixture of amino-acids which was free from sulphur, thus indicating the absence of methionine from the precipitate. Further fractionation according to Baptist & Robson [1940] yielded 90 mg. of a picrolonate and 320 mg. of crude leucine. The picrolonate after decomposition yielded 30·5 mg. of phenylalanine. Found: N, 8·35%; $C_{11}H_{11}O_2N$ requires N, 8·48%; $[\alpha]_D = -33·5°$ (Baptist & Robson [1940] give $[\alpha]_D = -35°$). The crude leucine fractions were combined and recrystallized: (Found: N, 10·5%. $C_6H_{13}O_2N$ requires N, 10·7%. $[\alpha]_D$ in 5% HCl $= +15·2°$.) The value of the optical rotation indicates that *iso*leucine was almost absent.

Basic fraction. Potato extract (4500 ml.) was treated with lead acetate as above described. Lead was removed in the usual manner and the solution after concentration to 1500 ml. was treated with *d*-tartaric acid to remove potassium. The potassium hydrogen tartrate precipitate contained practically no N. Excess of phosphotungstic acid was added after the solution had been concentrated. After 2 days at 0° the precipitate was filtered off and decomposed with baryta. Precipitation with phosphotungstic acid and decomposition with baryta were repeated in order to remove non-basic amino-acids. Silver sulphate was added at *p*H 3 and the precipitate was centrifuged and decomposed by 0·1 *N* HCl in 2% NaCl. This fraction, containing purines and no amino-acids (negative ninhydrin reaction), was worked up as described by Hoppe-Seyler & Thierfelder [1924]. Only adenine (47 mg.) was obtained in the form of its picrate of M.P. 282°; this was converted into the hydrochloride and the free base which was analysed. (Found: N, 51·3%. $C_5H_5N_5$ requires N, 51·85%.) The main solution was freed from silver and arginine was estimated by the method of Vickery [1940]; 1·32 g. of arginine monoflavianate were obtained corresponding to 0·471 g. of arginine; colorimetric estimation by the method of Jorpes & Thorén [1932] gave a value of 0·571 g. from which it appears likely that small amounts of other monosubstituted guanidines may have been present. The mother liquors from the arginine flavianate preparation were combined and flavianic acid was removed. Precipitation with silver and baryta yielded a small precipitate which, on decomposition, gave no Pauly reaction and contained very little N. Another experiment where the preliminary removal of arginine by flavianic acid was omitted and the Kossel-Vickery procedure was followed also gave no tests for histidine. Silver and baryta were now removed. A very weak ninhydrin reaction indicated that very little or no lysine was present. Further fractionation according to Schulze yielded 0·20 g. of trigonelline and 0·4 g. of choline. We have been unable to isolate cadaverine which Yoshimura [1934] claimed to have isolated from potatoes.

Isolation of choline. Freshly prepared ammonium reineckate (2 g.) was added to

1000 ml. of potato extract which was freed from protein and had been brought to *p*H 13·0. The precipitate was left overnight in the dark at 0° and then collected, dissolved in the funnel with 50% acetone and recrystallized from water. The precipitate which now had the typical lustre of choline reineckate weighed 395 mg. corresponding to 97 mg. of choline. The reineckate was decomposed with silver sulphate and baryta, and the solution was evaporated to dryness after acidification with HCl. The residue was extracted with alcohol and excess chloroplatinic acid was added. The chloroplatinate was recrystallized from aqueous alcohol and weighed 160 mg. It had M.P. 241° and analysed correctly for choline chloroplatinate. (Found: N, 4·5%. $C_{10}H_{28}O_2N_2Cl_6Pt$ requires N, 4·54%.)

PART III

Nitrogen distribution in different varieties and in different layers of the potato tuber

Typical results for eleven samples of potatoes obtained through the generous assistance of Mrs McDermott of the Midland Agricultural College are recorded in Tables 4 and 5. The potatoes were grown on river gravel (medium loam) and manured with farmyard manure and I.C.I. fertilizer no. 5. The samples consisted of 15–20 tubers of different sizes which had been carefully cleaned but not peeled. The differences in total N on a dry weight basis are very considerable, but are less marked if calculated in terms of fresh weight or fresh weight minus starch and fibre. The relative proportions of insoluble N

Table 4. *Dry matter, total N and protein-N in different varieties of potato*

Variety	Dry matter %	Total N % of dry weight	Total N % fresh weight	Protein-N % of total N	Protein % of fresh weight
Majestic 1	17·83	1·695	0·307	39·7	0·738
King Edward 1	19·67	1·825	0·359	44·5	0·992
Golden Wonder	21·96	1·387	0·3045	63·7	1·20
Doone Star	21·5	1·51	0·339	50·7	1·07
Great Scot	18·9	1·613	0·303	48	0·899
Redskin	16·37	1·949	0·319	37·5	0·694
Kerr's Pink	16·82	1·479	0·238	58	0·856
British Queen	19·15	1·59	0·304	44·5	0·837
Dunbar Standard	20·37	1·159	0·236	44·9	0·657
Arran Banner	18·99	1·652	0·314	37	0·719

Table 5. *Distribution of soluble N in different varieties of potato*

Variety	Protein-N	Asparagine-N	Glutamine-N	Glutamine / Asparagine ratio	α-Amino-acid-N	Basic N
Majestic 1	30·2	25·5	14·5	0·51	9·4	23·0
Majestic 2	28·5	27·0	14·8	0·55	11·5	21·0
Majestic 3	29·5	26·0	15·1	0·58	10·8	22·1
King Edward 1	34·5	16·0	17·5	1·1	12·8	23·9
King Edward 2	35·5	15·8	18	1·16	12·7	22·1
King Edward 3	36·0	18	16·8	0·93	—	—
Golden Wonder	51·2	11·8	7·6	0·64	7·3	23·7
Doone Star	39·7	17·7	12·6	0·71	6·4	26·7
Great Scot	37·1	19·0	12·4	0·65	10·5	27·7
Redskin	28·0	22·4	19·7	0·86	—	—
Kerr's Pink	46·7	13·9	8·7	0·63	—	—
British Queen	43·8	19·6	13·3	0·68	—	—
Dunbar Standard	34·7	20·4	12·8	0·60	9·5	20·4
Arran Banner	32·2	21·5	14·7	0·68	—	—

The figures represent % of total N.

The samples of varieties Majestic and King Edward 2 and 3 were grown near Cambridge, whilst all others were grown at Sutton Bonnington.

varied between about 8 and 12·5% of the total N and averaged about 0·33 g./1000 g. of fresh potatoes. In the soluble fraction the relative proportions of protein varied approximately between 25 and 50%; samples having a high soluble protein content also seem to have a high content of insoluble N. The total protein content in Table 4 is calculated on the assumption that the insoluble nitrogenous material is protein and that the average N content of the protein is 16·1%. Samples having a high relative protein content are low in amides and *vice versa* so that the sum of protein and amides is fairly constant, i.e. about 70%. N due to α-amino-acids other than amides was found in only small amounts; it varied between 6 and 12%.

It cannot be claimed that the values found for these samples necessarily represent entirely varietal characteristics. The potatoes, although grown under the same environmental conditions, may have been lifted at different stages of maturity; moreover the experiment was not sufficiently controlled for definite conclusions to be drawn. It does seem however that variety is an important factor in determining the N distribution of the tuber. Thus samples of varieties Majestic and King Edward grown in the fens, near Cambridge, and at Sutton Bonnington gave analyses which differed but little; all Majestic had much smaller relative protein values than any King Edwards examined. More striking however is the difference in the glutamine-asparagine ratios, which fell in variety Majestic between 0·55 and 0·65, whilst in variety King Edward it was always either close to or slightly above 1. It appears probable therefore that certain chemical estimations such as the estimation of the relative content of soluble protein and the determination of the glutamine-asparagine ratio can be used to characterize varieties. It may also be possible to select certain varieties having a high protein content for particular purposes such as animal feeding.

N distribution in the different layers of the potato

When potatoes are peeled and cooked a fairly large proportion of the outer layers is discarded. It was therefore considered interesting to examine how the different nitrogenous fractions are distributed over the tuber. If a potato is cut longitudinally three different parts can clearly be distinguished. Apart from the pigmented skin the cortex is divided from the pith or medulla by the bundle zone, which reaches the skin near the eyes. It has been known for some time that the water content is highest in the skin, decreases sharply in the cortex and reaches a second maximum in the inner medulla, whilst starch shows the reverse behaviour, i.e. it is highest in the inner cortex or outer medulla [Glynne & Jackson, 1919]. Not very much is known about the distribution of the different nitrogenous fractions; Rathsack [1935] states that soluble N and amino-N behave similarly to total N, whilst Cook [1921] found more insoluble N in the skin than in other parts of the tuber.

Two potato varieties, Majestic and King Edward, were used for these experiments (Tables 6 and 7). Ten tubers of different size were carefully washed and scraped and dissected into three or four parts respectively, which were weighed separately for each tuber. The skin amounted to 1·5–5% of the total fresh weight, according to the size of the tuber. The cortex was about 35–45% of the fresh weight. The division between outer and inner medullas was arbitrary. Samples of each layer were pooled, dried and ground as described in Part I. Table 6 shows that the distribution of the different nitrogenous fractions varies considerably in the different layers. Total N is high in the peel, decreases sharply in the cortex and rises again towards the pith, thus confirming the earlier work. The high N content of the peel is due to its high contents of insoluble nitrogenous matter and of asparagine. The insoluble N is mainly concentrated in the outer layers and falls nearly to zero towards the pith. The soluble protein, amino-acid-N and basic N are not quite evenly distributed throughout the tuber but the differences seem insignificant, particularly since the distribution of soluble protein varies in the two samples investigated.

In the skin the concentrations of all these constituents appear to be low. Very striking is the unequal distribution of glutamine and asparagine over the tuber. The cortex and peel store most of their amide in the form of asparagine, whilst glutamine is mainly found in the medulla. This unequal distribution may be interpreted as an indication of local differences in biological function of the various parts of the tuber and may be accompanied by similar differences in the distribution of sugars and organic acids.

The results show clearly that the N distribution varies considerably in different varieties and it is therefore very likely that their nutritive values will vary accordingly. An accurate assessment of the biological values of the different fractions on the basis of purely chemical data is however impossible; for instance nothing is known about the availability of the insoluble nitrogenous material to animals. Amides too, although not related to essential amino-acids, may be of some value in supplementing the N of the protein. It may also be mentioned that in cooked vegetables practically all the glutamine will be converted into pyrrolidone carboxylic acid which, if ingested in large amounts, may be excreted unchanged [Bethke & Steenbock, 1923]. The experiments on local distribution of N in the tuber indicate that a fairly large proportion of the total N is lost on peeling, although its biological value is uncertain.

Table 6. *N distribution in different layers of variety Majestic*

	Peel	Cortex	Outer medullary	Inner medullary
Fresh weight of each layer as % of total fresh weight	5·88	33·01	35·39	25·67
Dry weight of layer as % of its fresh weight	15·0	21·7	22·2	17·2
Total N	0·531	0·33	0·373	0·380
Soluble N	0·405	0·297	0·351	0·3705
Insoluble N	0·126	0·033	0·022	0·0095
Soluble protein-N	0·0626	0·096	0·104	0·083
Total protein-N	0·1886	0·122	0·126	0·107
Soluble non-protein-N	0·343	0·205	0·244	0·286
Total amide-N	0·237	0·101	0·137	0·179
Asparagine-N	0·1786	0·0699	0·085	0·1092
Glutamine-N	0·0587	0·027	0·0533	0·060
α-Amino-acid-N (less amides)	0·0182	0·040	0·0449	0·0396
Basic N	0·0634	0·0734	0·0832	0·0670
$\frac{\text{Glutamine}}{\text{Asparagine}}$ ratio	0·326	0·376	0·639	0·641

The N values are expressed as % of the total fresh weight of the layer.

Table 7. *Distribution of soluble N in King Edward potatoes*

	Peel	Cortex	Medulla
Protein-N	39 (15·4)	29 (32·4)	47 (26·7)
Asparagine-N	31·9 (44·1)	21 (24·2)	16·5 (26·5)
Glutamine-N	16·6 (14·5)	10·8 (9·1)	20·5 (16·3)
$\frac{\text{Glutamine}}{\text{Asparagine}}$ ratio	0·52 (0·326)	0·51 (0·376)	1·24 (0·640)
Total N as % of fresh weight	0·465 (0·531)	0·298 (0·33)	0·384 (0·375)

The values for soluble N are expressed as % of total soluble N.
The figures in brackets represent the corresponding figures for Majestic potatoes.

Summary

1. Methods are described for the estimation of different types of nitrogenous substances in plant extracts. It is shown particularly that the ninhydrin method is more reliable than the nitrous acid method for the estimation of α-amino-acid-N. A new method for the estimation of glutamine is proposed depending on the loss of carboxyl groups reacting with ninhydrin on heating.

2. A number of nitrogenous compounds have been isolated from potatoes such as asparagine, glutamine and arginine; these were shown to be present in fairly large amounts. Histidine, lysine and cystine appear to be absent. Choline was isolated and estimated by precipitation as reineckate in strongly alkaline solution.

3. Analyses are presented of the N contents and distributions in a number of domestic varieties and it is shown that varieties differ considerably in their protein and amide contents.

Thus the figures for total N were between 0·238 and 0·359% of the fresh weights and the relative proportion of protein varied even more. While Majestic potatoes contained about 29·5% of their total N in the form of protein and about 40% as amides, Golden Wonder potatoes had 51·2% of protein and 19·4% of amides.

4. It is also demonstrated that the nitrogenous compounds are not equally distributed over the tuber. Thus the insoluble nitrogenous material is mainly present in the skin and cortex. Glutamine is mainly stored in the inner layers, whilst the outer layers are richer in asparagine. The bearing of these results on the biological value of the potato N is briefly discussed.

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77. The Effect of Amino-acids on Phosphate Transfer in Muscle Extract*

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Lohmann [1934] demonstrated in vertebrate muscle extract phosphate transfer from creatinephosphate to adenylic acid resulting in adenylpyrophosphate and creatine. Ostern, Baranowski & Reis [1935], D. M. Needham & van Heyningen [1935], and Meyerhof & Lehmann [1935] all showed in vertebrate muscle extract phosphate transfer from adenylpyrophosphate to creatine resulting in creatinephosphate and adenylic acid. Meyerhof & Lehmann [1935] also reported phosphate transfer from adenylpyrophosphate to arginine in invertebrate muscle extract.

The two directions of phosphate transfer were investigated more closely by Lehmann [1935; 1936] who showed that the same equilibrium point was reached from both sides; it depended on *p*H. Alkalinity favoured phosphagen synthesis, neutrality and acidity favoured the formation of adenylpyrophosphate. The reversible transfer of 2 mol. phosphate between 1 mol. adenylic acid and 2 mol. creatine was shown not to be a trimolecular reaction but could be split up into two bimolecular reactions in which adenosine diphosphate (a substance discovered by Lohmann [1934] in crayfish) figured in vertebrate muscle also, as an intermediate between adenylpyrophosphate and adenylic acid.

Lehmann [1935] reported that vertebrate muscle extract phosphorylated only creatine and invertebrate muscle extract transferred phosphate only to arginine. Baldwin & D. M. Needham [1937] showed that those invertebrates which according to J. Needham, D. M. Needham, Baldwin & Yudkin [1932] contained both phosphagens, also had both the enzymes needed for phosphorylating creatine and arginine. In the work now reported we sought to find out whether there was competitive inhibition of creatine phosphorylation by arginine in vertebrate muscle extract or of arginine phosphorylation by creatine in muscle extract of invertebrates. We found however that arginine did not inhibit creatine phosphorylation but accelerated and even initiated it under certain conditions. Various other reactions, most of them involving phosphate transfer, were investigated, and the effect on them of arginine and other compounds was studied.

Methods

Enzymes used. The enzyme used was rabbit muscle extract prepared according to Meyerhof [1926]. To investigate the transfer of phosphate from adenylpyrophosphate to creatine and arginine, or the phosphorylation of adenylic acid from a phosphate donator, conditions had to be established in which enzymic side reactions were abolished as much as possible. They were mainly dephosphorylation of adenylpyrophosphate to inorganic phosphate and adenylic acid by adenylpyrophosphatase and deamination of adenylic acid to NH_3 and inosinic acid by adenylic acid deaminase. The activity of adenylpyrophosphatase was mainly abolished by letting the extract stand in the ice chest for several days or weeks. Combination of ageing with prolonged dialysis led to complete inactivation of adenylpyrophosphatase [Lehmann, 1935]. These facts were tested again and confirmed, since experiments on phosphate transfer by Lehmann & D. M. Needham [1937] had been criticized on this point, and it was claimed that one could never obtain complete destruction of adenylpyrophosphatase by ageing and dialysis only. The absence of adenylpyrophosphatase was demonstrated in the present work in each extract used for a series of experiments and checked again from time to time. The interference caused by adenylic acid deaminase could be completely disregarded in those of our experiments which were carried out at *p*H 10, at which reaction the deaminase is inactive [Lehmann, 1935]. In the experiments at a neutral or slightly alkaline reaction advantage was taken of the observation of G. T. Cori,

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Colowick & C. F. Cori [1939] that adenylic acid deaminase was removed from an aged extract by filtering. We confirmed these findings.

The extracts were aged by leaving at +4° for at least 5 days. Toluene was added. If it is not specially mentioned otherwise they also underwent dialysis through a cellophane membrane. The membrane was washed previously with dilute HCl to remove traces of Cu. The dialysis was carried out for a period of at least 48 hr. at +4°. The dialysis fluid was frequently changed and was in turn distilled water and 0·45% KCl. The last dialysis was always performed against 0·45% KCl.

Compounds used. The phosphorylated compounds used were prepared by us except the adenylic acid which was obtained from a Lwow (Poland) factory, and the hexosediphosphate which was the product of a U.S. firm. The adenylpyrophosphate was prepared by a modification of Lohmann's method [Lohmann, 1931] which was communicated to us by Dr D. M. Needham. Later we also used the modifications recommended by Kerr [1939]. Adenosinediphosphate was prepared from adenylpyrophosphate by means of dilute myosin. This, according to a personal communication of Dr K. Bailey, only dephosphorylates adenylpyrophosphate but does not attack adenosinediphosphate—the enzyme responsible for dephosphorylation of adenosinediphosphate being the myokinase [Kalckar, 1942]. The myosin was given to us by Dr J. Needham and Dr Shen. Creatinephosphate was prepared by the enzymic phosphorylation of creatine from phosphoglyceric acid [Lehmann, 1935] and phosphoglyceric acid was prepared from glucose and phosphate in fluoride-poisoned yeast extract [Meyerhof & Kiessling, 1933]. All the phosphate esters were used as the K or Na salts.

Most of the amino-acids and related compounds used were commercial products. Dr Bach provided us with ornithuric acid, glycocyamine and citrulline. The cystine was obtained from Mr Cole and the α-ketoglutaric acid was prepared by Mr Williamson. Prof. Sir F. G. Hopkins gave us reduced and oxidized glutathione.

Experimental procedure

If it is not stated otherwise each sample contained 20% muscle extract, 0·13% Mg (as $MgCl_2$) and 0·1% $NaHCO_3$. The resulting mixture was just neutral or slightly alkaline (up to *p*H 7·6). In most experiments Na_2CO_3 was added to give a final concentration of $M/20$, resulting in a *p*H of about 10. If Na_2CO_3 was present, the various solutions were added in such order as to avoid the enzyme being at any moment in a Na_2CO_3 concentration of $> M/20$. Higher concentrations were apt to destroy the enzyme. Some inorganic phosphate was always present. The dialysed extract contained usually 1–2 mg. per 100 ml. P as inorganic phosphate and the Ba salt of the adenylpyrophosphate used contained 0·6–0·8% P as inorganic P. This resulted in the presence of 0·6–0·8 mg. per 100 ml. P as inorganic phosphate in that majority of the experiments in which adenylpyrophosphate and dialysed extract were used. All the compounds were, if necessary, neutralized beforehand. If they were buffers, their *p*H was adjusted to that of the experiments. Further particulars will be given in the text. At the end of the experiments trichloroacetic acid was added to give a final concentration of 5% and the extract used for analysis. The phosphate was measured colorimetrically using the modification of the method of Fiske & Subbarow [1925] given by Lohmann & Jendrassik [1926]. A standard of 200 μg. P in 25 ml. was used and if the sample to be estimated contained a much lower concentration, a measured amount of inorganic phosphate was added to make it comparable with the standard. The creatine phosphate was measured after Lohmann [1928] as the difference between the directly estimated phosphate (inorganic phosphate + phosphagen phosphate) and the true inorganic phosphate precipitable by ammoniacal Mg citrate [Mathison, 1909]. The determination of arginine phosphate followed the same principle, but owing to the greater stability of arginine phosphate against acid the 'direct' value was measured after 20 hr. previous incubation in $N/15$ HCl at 24°.

The easily hydrolysable phosphate of adenylpyrophosphate and adenosinediphosphate was measured as the increase in inorganic phosphate after 7 min. hydrolysis in N HCl at 100°. The change of 3-phosphoglyceric acid into 2-phosphopyruvic acid was observed by measuring the increase of inorganic phosphate after 60 min. hydrolysis in N HCl at 100° [Lohmann & Meyerhof, 1934]. The formation of triosephosphate from hexosediphosphate was followed by measuring the increase of inorganic phosphate after 20 min. incubation in N NaOH at room temperature [Meyerhof & Lohmann, 1934]. The free NH_3 was measured colorimetrically using Nessler's reagent, if necessary after a previous distillation in the Parnas apparatus.

All figures will be given for a volume of 10 ml. The volume employed in our experiments was usually 4 ml.

Transfer of phosphate from adenylpyrophosphate to creatine

The effect of arginine. Our original aim was to find if arginine inhibited creatine phosphorylation, but it showed itself to be an activator. This could be seen when the activity of the extract had diminished owing to prolonged dialysis. An extract which before dialysis transferred phosphate from adenylpyrophosphate to creatine in a few minutes, after dialysis needed 20–30 min. for final equilibrium. The experiments were performed under the same conditions each time and Mg was added to the dialysed extract. Arginine was seen to accelerate the transfer in feebly active extracts, though it did not change the final equilibrium (Table 1).

Table 1. *Activation of a weak extract by arginine*

Dialysed rabbit muscle extract, 21 days old. Adenylpyrophosphate containing 825 μg. transferable P. 7·5 mg. creatine. *p*H 10. 37°.

Time in min.	Creatine phosphate formed, μg. P: No addition	Creatine phosphate formed, μg. P: 75 mg. arginine
3	147	560
30	545	525

Table 2. *Reactivation of an inactive extract by arginine*

Dialysed rabbit muscle extract, 18 days old. Adenylpyrophosphate containing 825 μg. transferable P. 7·5 mg. creatine. *p*H 10. 37°.

Time in min.	Addition	Creatine phosphate formed, μg. P
1	—	15
1	75 mg. arginine	290
30	—	12
30	75 mg. arginine	395

Some of the extracts which had been dialysed longer became completely inactive and did not produce a transfer of phosphate even over a prolonged period. In such cases, arginine largely restored the transfer (Table 2).

The inactivity of the extract and the effect of arginine upon it were studied in two ways: (*a*) the reason for activation by arginine, whether it was a specific effect of the arginine molecule or of a particular part of it which it might share with other compounds; (*b*) whether changed conditions (*p*H, dialysis etc.) could restore activity to the extract.

1. *Comparison with the effects of other compounds*

To see whether the guanidine group of arginine was responsible for the activation, guanidine itself was tried as well as urea and NH_3 (Table 3) and also guanidoacetic acid (glycocyamine) (Table 4). These substances were inactive.

Table 3. *No influence of guanidine, urea and NH_3*

Dialysed rabbit muscle extract, 8 days old. Adenylpyrophosphate containing 875 μg. transferable P. 7·5 mg. creatine. *p*H 10. 33°. 10 min.

Addition	Creatine phosphate formed, μg. P
—	245
23 mg. guanidine	235
125 mg. urea	225
33 mg. NH_3	240

Table 4. *No influence of glycocyamine*

Dialysed rabbit muscle extract, 30 days old. Adenylpyrophosphate containing 825 μg. transferable P. 7·5 mg. creatine. *p*H 10. 33°. 5 min.

Addition	Creatine phosphate formed, μg. P
—	0
25 mg. glycocyamine	0
25 mg. arginine	190
50 mg. arginine	200

The experiment with glycocyamine (Table 4) demonstrates that 25 mg. arginine are sufficient for full activation.

To test whether the α-aminovaleric acid part of the arginine molecule was responsible for its effect, citrulline was tried which differs from arginine only in that the imino group of guanidine is replaced by oxygen (Table 5), and ornithine (α:δ-diaminovaleric acid, Table 6).

Table 5. *Activation by citrulline*

Dialysed rabbit muscle extract, 7 days old. Adenylpyrophosphate containing 825 μg. transferable P. 7·5 mg. creatine. *p*H 10. 30°.

Time in min.	Creatine phosphate formed, μg. P: No addition	Creatine phosphate formed, μg. P: 50 mg. citrulline
5	125	300
20	280	388
40	306	375

Table 6. *Activation by ornithine*

Dialysed rabbit muscle extract, 11 days old. Adenylpyrophosphate containing 740 μg. transferable P. 7·5 mg. creatine. *p*H 10. 33°. 20 min.

Addition	Creatine phosphate formed, μg. P
—	0
12·5 mg. ornithine	80
25 mg. ornithine	185
50 mg. arginine	170

Of all the compounds used so far, only the amino-acids were found to be active regardless of whether a guanidine group was present or not. The question arose now which particular group of the aminovaleric acid was the active one. Ornithine was thus compared with ornithuric acid in which the two amino groups are blocked with benzoic acid. No influence of ornithuric acid could be demonstrated (Table 7).

Table 7. *Activation by ornithine, no activation by ornithuric acid*

Dialysed rabbit muscle extract, 48 days old. Adenylpyrophosphate containing 830 μg. transferable P. 7·5 mg. creatine. *p*H 10. 35°. 5 min.

Addition	Creatine phosphate formed, μg. P
—	25
M/60 ornithine	365
M/60 ornithuric acid	25

Table 8. *Activation by glutamic acid, no activation by α-ketoglutaric acid*

Dialysed rabbit muscle extract, 17 days old. Adenylpyrophosphate containing 1450 μg. transferable P. 7·5 mg. creatine. *p*H 10. 35°. 5 min.

Addition	Creatine phosphate formed, μg. P
—	35
100 mg. glutamic acid	680
100 mg. α-ketoglutaric acid	30

The last experiments made it almost sure that a free amino group was the effective group, and comparison with other α-amino-acids showed that they all activated. The essential nature of the α-amino group could be demonstrated by replacing it by O, OH or H. Thus alanine was active but not pyruvic, lactic or propionic acid. Glycine was effective but not acetic acid; aspartic acid but not succinic or fumaric acid. In Table 8 a comparison is given of the effects of glutamic acid and of α-ketoglutaric acid.

The various amino-acids were compared with one another. Table 9 shows that the diaminohexoic acid lysine was not more active than the monoamino-*iso*hexoic acid leucine, if the usual concentration of activators was used.

Table 9. *Comparison of lysine and leucine*

Dialysed rabbit muscle extract, 6 days old. Adenylpyrophosphate containing 1450 μg. transferable P. 7·5 mg. creatine. *p*H 10. 34°. 5 min.

Addition	Creatine phosphate formed, μg. P
—	37
M/15 lysine	365
M/15 leucine	380

Table 10. *Comparison of* d(−)*-alanine and* l(+)*-alanine*

Dialysed rabbit muscle extract, 5 days old. Adenylpyrophosphate containing 1450 μg. transferable P. 7·5 mg. creatine. *p*H 10. 34°. 5 min.

Addition	Creatine phosphate formed, μg. P
—	65
60 mg. *d*(−)-alanine	430
60 mg. *l*(+)-alanine	455

There was no difference as regards their activating effects between *d*(−)-alanine and *l*(+)-alanine (Table 10).

Cysteine was still active at concentrations which were too low for other amino-acids to show an effect (Table 11).

The higher activity at low concentrations of cysteine suggested that sulphydryl had an effect similar to that of the α-amino group. This was confirmed by the activation brought about by thiolacetic acid (Table 12).

The tripeptide glutathione was more active than cysteine (Table 13). The action of S—S compounds like cystine and oxidized glutathione was not inhibitory. They activated slightly, but much less than their reduced forms. This may have been due to their forming some sulphydryl under the conditions of our experiment, and also to their containing the activating α-amino group. They did however diminish the influence of activating amino-acids, regardless of whether these contained an SH group or not (Table 13).

Table 11. *Comparison of cysteine with glycine and lysine*

Dialysed rabbit muscle extract, 7 days old. Adenylpyrophosphate containing 1450 μg. transferable P. 7·5 mg. creatine. *p*H 10. 35°. 5 min.

Molarity of addition	Creatine phosphate formed, μg. P		
—	30		
	Cysteine	Glycine	Lysine
1/30	—	295	—
1/60	260	270	260
1/120	170	35	25
1/240	155	—	20

Table 12. *Activation by thiolacetic acid**

Dialysed rabbit muscle extract, 32 days old. Adenylpyrophosphate containing 1450 μg. transferable P. 7·5 mg. creatine. *p*H 10. 35°. 5 min.

Addition	Creatine phosphate formed, μg. P
—	120
38 mg. thiolacetic acid	305

* Much sulphydryl in the experiments interfered with the phosphate estimation. The trichloroacetic extract was therefore treated with chloramine T. A slight excess of the calculated amount needed was added and the precipitate which appeared was filtered off. We are obliged to Mr J. R. Bendall for advice on this method.

Table 13. *Activation by reduced glutathione (GSH): the effect of oxidized glutathione (GS-SG) and cystine on the activation by other amino-acids*

Dialysed rabbit muscle extract, 37 days old. Adenylpyrophosphate containing 830 μg. transferable P. 7·5 mg. creatine. *p*H 10. 35°. 5 min.

Additions	Creatine phosphate formed, μg. P	Increase in creatine phosphate formed, μg. P	Additions	Creatine phosphate formed, μg. P	Increase in creatine phosphate formed, μg. P
—	155	—	*M*/50 cysteine, *M*/50 cystine	220	65
M/50 GSH	525	370	*M*/20 glycine	455	300
M/80 GS-SG	195	40	*M*/20 glycine, *M*/80 GS-SG	195	40
M/50 GSH, *M*/80 GS-SG	435	280	*M*/20 glycine, *M*/50 cystine	200	45
M/50 cysteine	280	125	*M*/30 leucine	385	230
M/50 cystine	190	35	*M*/30 leucine, *M*/50 cystine	185	30

Simple primary amines were effective, but acetamide, the only amide we tested, had no influence. In Table 14 a comparison is made between acetamide and ethylamine.

Table 14. *No influence of acetamide: activation by amines*

Dialysed rabbit muscle extract, 21 days old. Adenylpyrophosphate containing 850 μg. transferable P. 7·5 mg. creatine. *p*H 10. 35°. 5 min.

Addition	Creatine phosphate formed, μg. P	Addition	Creatine phosphate formed, μg. P
—	0	α-phenylethylamine	160
M/12 acetamide	0	*M*/35 histamine	455
M/12 ethylamine	422		

In Table 15 are recorded substances which were tested for their effect on transfer of phosphate in dialysed muscle extract at *p*H 10.

Table 15. *Compounds tested for their effect upon phosphate transfer in dialysed muscle extract at alkaline* p*H*

Effective	Not effective
Glycine, thiolacetic acid	Acetamide, acetic acid
d(−)-Alanine, *l*(+)-alanine, cysteine	Pyruvic acid, lactic acid, propionic acid
Aspartic acid	Succinic acid, fumaric acid
Glutamic acid	α-Ketoglutaric acid
Arginine, citrulline, ornithine	Urea, guanidine, creatinine, glycocyamine
Leucine, lysine	—
Histidine	—
Glutathione (GSH)	—
Methylamine, ethylamine	Ammonia, choline
α-Phenylethylamine, piperidine, histamine	—

2. *Conditions necessary for activation*

(*a*) *Alkaline reaction.* No activation by amino-acids was found at neutrality. To obtain time curves of the enzyme action, diluted extracts were used, but no rise in the velocity of phosphate transfer could be observed when amino-acids were present. Table 16 shows an experiment where the extract was not 20% of the total volume as usual in our experiments, but only 0·3%. It will be seen that at *p*H 7·5 the equilibrium was much in favour of adenylpyrophosphate, and that it was necessary to add a great surplus of creatine to obtain an equilibrium point which involved a significant shift of phosphate.

Table 16. *No influence of amino-acids at low* p*H*

Dialysed rabbit muscle extract, 76 days old. Adenylpyrophosphate containing 820 μg. transferable P. 34°.

Time in min.	*p*H	Extract % of total vol.	Creatine, mg.	Addition	Creatine phosphate formed, μg. P
15	10	20	7·5	—	48
15	10	20	7·5	50 mg. glycine	450
15	7·5	0·3	7·5	—	63
15	7·5	0·3	7·5	50 mg. glycine	63
1	7·5	0·3	100	—	52
1	7·5	0·3	100	50 mg. glycine	30
1	7·5	0·3	100	50 mg. arginine	40
1	7·5	0·3	100	50 mg. thiolacetic acid	65
15	7·5	0·3	100	—	220
15	7·5	0·3	100	50 mg. glycine	230
15	7·5	0·3	100	50 mg. arginine	195
15	7·5	0·3	100	50 mg. thiolacetic acid	235

The fact that at *p*H 7·5 large amounts of creatine had to be used made it possible that the increase in creatine and not the lower *p*H was responsible for the insensitivity of the reaction towards amino-acids. Large amounts of creatine might have been able to take on the role of the amino-acids. In testing this point we had to take into account that creatine, unlike the amino-acids, shifted the equilibrium point and large amounts of it would be expected to increase the phosphate transfer at neutral and at alkaline reaction. Table 17 shows however that an added excess of creatine at an alkaline reaction did not

Table 17. *Activation by glycine in the presence of excess of creatine*

Dialysed rabbit muscle extract, 14 days old. Adenylpyrophosphate containing 1000 μg. transferable P. *p*H 10. 35°.

Time in min.	Extract % of total vol.	Creatine, mg.	Addition	Creatine phosphate formed, μg. P
5	20	7·5	—	125
5	20	7·5	25 mg. glycine	290
5	20	75	—	325
1	1	7·5	—	150
1	1	7·5	25 mg. glycine	250
5	1	75	—	300
5	1	75	25 mg. glycine	480

prevent the amino-acids from exerting their influence. Compared with low amounts of creatine an excess had a beneficial influence, but this could be greatly improved upon by addition of glycine. The absolute proof of the inability of creatine to replace amino-acids in their activation will be given when it will be shown that it did not activate the phosphorylation of arginine (see Table 21).

(*b*) *Dialysis.* Undialysed extract was more active than its dialysed equivalent and could not be improved by addition of glycine (Table 18). Undialysed extract seemed thus

to contain a factor which was lost on dialysis and which could be replaced by an amino-acid. This factor was heat-stable, and boiled undialysed extract could be used for the activation of dialysed extract (Table 18). (The boiled extract was prepared by 15 min. heating on a boiling water bath. It was seen to be inactive by itself. This had to be tested as Curtius & Ohlmeyer [1938] have shown that a number of enzymes transferring phosphate are not completely destroyed by short boiling.) The activating principle was present in the dialysate but not in the ash of the dialysate. The dialysate was prepared by dialysing successively four equal samples of 1 vol. muscle extract for 4 hr. against the same sample of 4 vol. water. The dialysate was then concentrated and contained inorganic phosphate = 30 mg. per 100 ml. P, as compared with 50 mg. per 100 ml. P in the undialysed extract. The similarity of the actions of the effective principle in undialysed muscle extract and of the amino-acids added to dialysed extract is borne out by the fact that cystine inhibited the undialysed extract. It was shown previously (Table 13) that it did not inhibit dialysed extract, but counteracted the activation by added amino-acids.

Table 18. *No influence of amino-acids on undialysed extract. Activation of dialysed extract by boiled undialysed extract and dialysate*

Rabbit muscle extract, 14 days old. Adenylpyrophosphate containing 840 μg. transferable P. 7·5 mg. creatine added. *p*H 10. 35°. 6 min.

Enzyme	Addition	Creatine phosphate formed, μg. P
20% dialysed extract	—	125
20% dialysed extract	50 mg. glycine	350
20% undialysed extract	—	665
20% undialysed extract	50 mg. glycine	640
20% undialysed extract	50 mg. cystine	195
—	25% boiled undialysed extract	0
20% undialysed extract	25% boiled undialysed extract	467
20% dialysed extract	25% dialysate	280
20% dialysed extract	5% dialysate	150
20% dialysed extract	Ash equivalent to 25% dialysate	135

Transfer of phosphate from adenosinediphosphate to creatine

The amino-acid effect was not restricted to the shift of one phosphate group of adenylpyrophosphate to creatine; an influence was also observed on the transfer of the second phosphate group. Using adenylpyrophosphate as a donator we rarely obtained a shift of more than 50% of the transferable phosphate. Experiments were therefore carried out in which adenosinediphosphate served as the phosphate donator. The effect of amino-acids on phosphate transfer from adenosinediphosphate to creatine is shown in Table 19.

Table 19. *Activation of phosphate transfer from adenosinediphosphate to creatine*

Dialysed rabbit muscle extract, 38 days old. Adenyldiphosphate containing 625 μg. transferable P. 7·5 mg. creatine. *p*H 10. 35°. 10 min.

Addition	Creatine phosphate formed, μg. P
—	50
50 mg. glycine	413
37 mg. cysteine	438

Table 20. *Activation of phosphate transfer from creatine phosphate to adenylic acid*

Dialysed rabbit muscle extract, 28 days old. 1·25% of total volume. Creatine phosphate containing 600 μg. transferable P. 7·5 mg. adenylic acid. 36°. 1 min.

*p*H	Addition	Transfer of phosphate μg. P
8	—	62
8	30 mg. cysteine	325
8	25 mg. glycine	200
10	—	42
10	25 mg. glycine	93

Transfer of phosphate from creatine phosphate to adenylic acid

The transfer of phosphate from creatine phosphate to adenylic acid is lowest at an alkaline reaction. Thus high *p*H, which was most suitable for demonstrating the amino-acid effect, was most unsuitable for the reaction itself. We succeeded however with a very inactive extract, used in great dilution (1·25% of the total volume instead of the usual 20%), in demonstrating an activation at *p*H 8 and a small but definite one even at *p*H 10 (Table 20).

Transfer of phosphate from adenylpyrophosphate to arginine

An amino-acid effect on arginine phosphorylation in crab muscle extract was also observed. Glycine and cysteine greatly activated a crab muscle extract, which was itself completely inactive at *p*H 10 (Table 21). Creatine had no effect, as was to be expected, for its amino-group is substituted.

Table 21. *Activation of phosphate transfer from adenylpyrophosphate to arginine*

Dialysed crab muscle extract, 3 days old. Adenylpyrophosphate containing 800 μg. transferable P. 7·5 mg. arginine. *p*H 10. 37°. 5 min.

Addition	Arginine phosphate formed, μg. P
—	0
50 mg. creatine	0
50 mg. glycine	245
37 mg. cysteine	278

Table 22. *No inhibition of arginine phosphorylation by creatine*

Dialysed crab muscle extract, 8 days old. Adenylpyrophosphate containing 1250 μg. transferable P. 75 mg. arginine. *p*H 7·5. 38°. 15 min.

Addition	Arginine phosphate formed, μg. P
—	260
50 mg. creatine	243

The same extract which was inactive at *p*H 10 worked perfectly at *p*H 7·5. For the same reasons that required addition of more creatine at *p*H 7·5, more arginine had to be used at this reaction than would have been needed at *p*H 10. Table 22 shows that the improved transfer of phosphate was not inhibited by creatine; thus just as arginine was no competitive inhibitor of creatine phosphorylation, creatine was no competitive inhibitor of the phosphorylation of arginine.

Formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid

The formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid involves an intramolecular shift of phosphate. An effect of amino-acids on the reaction was demonstrated (Table 23). The enzyme system involved was—unlike the one which phosphorylated creatine—almost inactive at *p*H 10 even when undialysed extract was used. Again no amino-acid effect could be seen at a neutral reaction.

Table 23. *Activation of the formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid*

Dialysed rabbit muscle extract, 5 days old. 40% of the total volume. Phosphoglyceric acid containing 4000 μg. P. 25°. 20 min.

*p*H	Addition	Phosphopyruvic acid formed, μg. P
7	—	640
7	80 mg. glycine	640
10	—	0
10	80 mg. glycine	160

Table 24. *Influence of glycine on the speed of formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid*

Dialysed rabbit muscle extract, 11 days old. 40% of the total volume. Phosphoglyceric acid containing 4000 μg. P. *p*H 8·5. 25°.

Time in min.	Phosphopyruvic acid formed, μg. P		Conversion %	
Addition	—	80 mg. glycine	—	80 mg. glycine
15	0	520	0	13
30	460	760	11·5	19
60	968	948	24	23

The equilibrium between phosphoglyceric acid and phosphopyruvic acid is reached at room temperature when 75–80% of the total P belongs to phosphoglyceric acid and 25–20% to phosphopyruvic acid [Lohmann & Meyerhof, 1934]. We thus investigated again whether the amino-acid effect shifted the equilibrium or whether it only increased the speed of its attainment. Table 24 shows that the equilibrium itself was not changed by glycine, but that the velocity was increased. It will be remembered that the same was seen when the equilibrium between adenylpyrophosphate and creatine phosphate was investigated.

Formation of triosephosphate from hexosediphosphate

The question arose whether the amino-acid effect applies to reactions of phosphoric esters in general or whether it is confined to reactions involving phosphate transfer. We therefore investigated whether amino-acids affect the formation of triosephosphate from hexosediphosphate, a reaction which does not involve a shift of phosphate [Meyerhof & Lohmann, 1934]. No influence could be observed (Table 25).

Table 25. *No influence of glycine on triosephosphate formation from hexosediphosphate*

Dialysed rabbit muscle extract, 14 days old. Hexosediphosphate containing 4000 μg. P. *p*H 8. 36°.

Time	Concentration of extract %	Triosephosphate formed, μg. P		Conversion, %	
	Addition ...	—	40 mg. glycine	—	40 mg. glycine
3	40·00	1020	1020	26	26
6	40·00	1020	1040	26	27
20	40·00	1000	1040	25	27
3	0·40	875	800	22	20
15	0·40	1000	1020	25	26
1	0·20	600	630	15	16
12	0·20	980	940	25	24
3	0·04	250	100	6	3
15	0·04	510	560	13	14

The influence of amino-acids on the solubility of slightly soluble salts

In most of our experiments which were carried out at an alkaline reaction the Mg was added to the muscle extract first and the adenylpyrophosphate and the Na_2CO_3 followed afterwards. In some the Mg, adenylpyrophosphate and Na_2CO_3 were first mixed, and afterwards the muscle extract was added. In the latter case a precipitation of Mg adenylpyrophosphate and $MgCO_3$ occurred. This precipitation could not be seen in the first procedure even when filtered and clear extract was used, this lack of visible precipitation was obviously due to the presence of colloids. In the second case the precipitate of Mg adenylpyrophosphate and $MgCO_3$ was completely dissolved in the samples where amino-acids were added. This could not have been due to the buffering effect of the amino-acids as they were adjusted to the alkaline reaction.

The effect of amino-acids was not restricted to the solubility of Mg adenylpyrophosphate and $MgCO_3$, but could also be observed with Mg phosphate and Ca salts.* If for instance two samples of 1 ml. of $M/10$ $MgHPO_4$ in $N/20$ HCl were treated with three drops of N NaOH, a precipitate of Mg phosphate appeared. To one sample 2 ml. of water were added and to the other 2 ml. of a 20% glycine solution which had been adjusted to *p*H 8·5 (9 mol. glycine : 1 mol. NaOH). The second sample became a clear solution. Phytin which is a commercial product consisting of a mixture of Ca and Mg phytates is sparingly soluble in pure water, but 100 ml. of M glycine solution dissolve up to 0·5 g.

Fig. 1 shows the effect of various glycine concentrations on the solubility of Mg phosphate, and Table 26 shows the effect of glycine on the solubility of Ca phosphate.

* The solubility of Ca oxalate is not altered by amino-acids and addition of excess Na oxalate will precipitate all Ca in a solution containing amino-acids and Ca salts of organic or inorganic phosphate.

The same compounds which activated phosphate transfer also had an effect on the solubility of sparingly soluble salts.

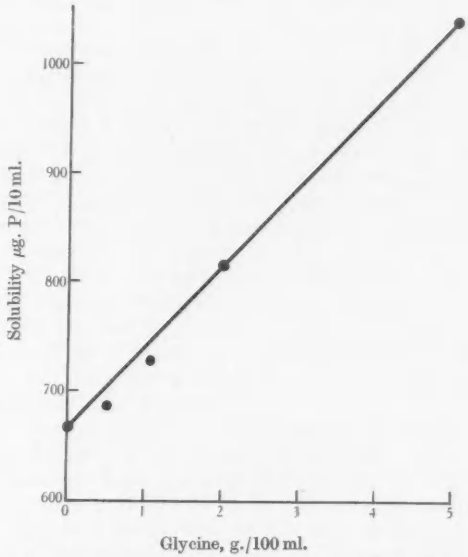

Fig. 1. The effect of glycine on the solubility of Mg phosphate ($MgHPO_4$, $3H_2O$).

Table 26. *Solubility of Ca phosphate in a glycine buffer at* pH *8·5 (9 mol. glycine: 1 mol. NaOH)*

Room temperature

Conc. of buffer	μg. P dissolved in 10 ml.
M/10	206
M/5	250
M/2	450
M	504

Table 27. *Different effects of glycine and cysteine on the solubility of Ca phosphate ($CaHPO_4$). Neutral* pH

Room temperature

Conc. of amino-acid	μg. P dissolved in 10 ml.	
—	118	
	Glycine	Cysteine
M/10	145	180
M/5	176	292

Thus glycine and thiolacetic acid produced the effect but not acetamide and acetate. Amines like piperidine and histamine redissolved sparingly soluble salts, but urea, guanidine etc. did not do so. Cysteine was more effective than glycine (Table 27).

Discussion

The accelerating and activating effect of amino-acids on phosphate transfer in muscle extract is obviously due to their amino group. This is proved by the observation that amines were likewise effective, but not amino-acids or amines in which the amino group was substituted (ornithine and choline). The SH group seems to act similarly on phosphate transfer, as is indicated by the effect of thiolacetic acid. Cysteine which contains both an amino and an SH group was found to be active at lower concentrations than S-free amino-acids. The fact that at alkaline reaction undialysed extract is more active than its dialysed equivalent may be due at least partly to the loss of amino-acids and related

compounds. It has been demonstrated that dialysed extracts could be activated by addition of boiled undialysed extract or dialysate. The behaviour of cystine points in the same direction. It inhibits undialysed extract but shows no effect on dialysed extract. If, however, the latter is activated by amino-acids, their effect can be suppressed by addition of cystine.

There are some facts in the literature which may possibly have a bearing on our findings. Boiled muscle extract was shown by Kendal & Stickland [1937] to contain activators other than the known coenzymes. Some effects are known of amino-acids on the activity of amylase, urease, lipase, phosphatase. Sumner & Hand [1928] protected urease from inactivation by adding amino-acids, and they give a long list of instances where previous authors found a protective action of amino-acids on enzymes; Bodansky [1936] in particular observed an activation of bone phosphatase by amino-acids which he also attributes to their protective function. However, the concentrations used by Bodansky were much below those employed by us.

Amino-acids are also often used to protect enzymes from metals. We could find no particular sensitivity of creatine phosphorylation towards Zn and Cu, the two metals which were most inhibitory in the formation of Robison ester from glycogen [Gill & Lehmann, 1939]. We found no appreciable inhibition of undialysed extract with $ZnSO_4$ up to *M*/400. There is always a certain danger of Cu contamination where cellophane tubes are used for dialysis. Thus Kubowitz & Lüttgens [1941] dialysed their cocarboxylase against a glycine solution to protect the enzyme against Cu. We washed our dialysis tubes with dilute HCl, and using the usual qualitative methods could find no Cu in our extracts. Moreover the transfer of phosphate from adenylpyrophosphate to creatine is inhibited only by relatively large amounts of Cu. In Table 28 the effect of high concentrations of Cu is demonstrated. It is reversed by glycine and the transfer is activated beyond the normal figure. However, Cu in the comparatively high concentration of *M*/40,000 had no effect on the reaction.

Table 28. *Inhibition by Cu and its suppression by glycine. No inhibition at* M/*40,000 Cu*

Dialysed rabbit muscle extract, 36 days old. 850 μg. transferable P of adenylpyrophosphate. *p*H 10. 36°.

Time in min.	Creatine, mg.	Added $CuSO_4$	Creatine phosphate formed, μg. P: No addition	Creatine phosphate formed, μg. P: 50 mg. glycine
3	60·	0	323	—
3	60	*M*/40,000	300	—
3	7·5	0	178	500
3	7·5	*M*/400	35	302
3	7·5	*M*/4000	105	440
3	7·5	*M*/40,000	185	—

There is a possibility of other metals being present which may have a specific inhibitory effect. However, if the amino-acid effect were simply a removal of metal it would be difficult to understand why it takes place only at an alkaline reaction. Furthermore the ash of the dialysate did not inhibit when added to muscle extract (Table 18).

It may be suggested that the effect of amino-acids on the solubility of sparingly soluble salts could well be connected with their activation of phosphate transfer. This would explain why no activation by amino-acids could be observed at a neutral reaction. Magnesium salts particularly are easily soluble at neutrality and therefore no increase in solubility would be necessary. The amino-acids may also influence the combination of the phosphate with the enzyme itself. This would agree with the fact that the amino-acids influence reactions involving phosphate transfer, but not for instance the formation of triosephosphate from hexosediphosphate.

The interaction of divalent ions, amino-groups, and phosphate, revealed in the experiments on solubility, may be a model for the activation of phosphatases by divalent ions such as Mg, Ca, Mn. These ions may link the phosphorus with the amino group of the enzyme molecule.

The higher solubility of sparingly soluble Ca salts in presence of amino-acids may be of importance for the intestinal absorption of these compounds. It has been shown that Ca, when taken by man as the soluble chloride, is largely converted into sparingly soluble $CaCO_3$ in the intestine [J. B. S. Haldane, R. Hill & Luck, 1923]. It is particularly remarkable that the solubility of Ca phytate is influenced by amino-acids. The question whether amino-acids play a part in the absorption of Ca and Mg has been taken up by one of us (H. L.) with Dr McCance and Dr Widdowson, and the influence of protein on Ca absorption in man could be demonstrated [McCance, Widdowson & Lehmann, 1942].

The effect of amino-acids particularly on the solubility of calcium salts may of course play a part in many physiological events.

The salutary effect of proteolytic enzymes on wound healing may be connected with it, a relation between calcium and cell growth being well known.

Plant seeds are particularly rich in protein and also in sparingly soluble calcium and magnesium salts. The breakdown to amino-acids may help to make the store of mineral salts available for the growing plant.

R. Hill & Lehmann [1941] observed that there was in the leaves of *Euonymus* a parallelism between iron and phosphorus contents. Experiments by the same authors on iron in various leaves showed that only a small proportion of it was 'available'. Our present observations demonstrate that it cannot be decided whether a salt is physiologically available in animal or plant tissues by testing its solubility in an aqueous or even acid extract. The extracts must contain the same compounds which might increase the solubility of the salts inside the cell. We could find no influence of glycine on the solubility of ferric salts. Of the compounds used in the present investigation both cysteine and thiolacetate increased considerably the solubility of ferric phosphate, ferric adenylpyrophosphate and even of ferric phytate at a neutral reaction. The soluble compound was purple, a sign that the stable thiol complex was formed. Citrate, preferably at a slightly alkaline reaction, showed a similar effect. Thus if amino-acids in general should play a role in making calcium and magnesium salts available for the growing plant, no exactly parallel mechanism can be assumed for ferric salts.

A soluble combination of the sparingly soluble $BaCO_3$ with amino-acids has previously been observed by Siegfried [1905]. He suggested that carbamino-acids were formed. Complex compounds of amino-acids with soluble salts such as $CaCl_2$ and $BaCl_2$ were found by Pfeifer & Modelski [1912; 1913] and crystallized by King & Palmer [1920]. Our findings are new in so far as they show that the addition of amino-acids raises the solubility of otherwise sparingly soluble salts, particularly at an alkaline reaction. A mutual effect on solubility of highly charged proteins or amino-acids and neutral salts such as NaCl and LiCl has been described by Cohn [1935]. It differs from our observations in its order of magnitude.

Summary

1. The effect of arginine on phosphorylation of creatine by adenylpyrophosphate has been studied in rabbit muscle extract. It increases the velocity of creatine phosphate formation, but does not shift the final equilibrium.

2. The effect of arginine has been compared with that of similar compounds (guanidine, glycocyamine, ornithine, ornithuric acid, citrulline). Only those containing an α-amino group were effective.

3. Amino-acids in general were tested and found to be effective. The corresponding keto-acids were ineffective.

4. Cysteine was still effective at $M/240$ but in the cases of glycine and lysine the activation disappeared between $M/60$ and $M/120$. Thiolacetic acid was effective.

5. Amines were effective but not acetamide.

6. The amino-acid effect could only be demonstrated at alkaline reaction, and on dialysed extract.

7. Dialysed extract could be activated by boiled undialysed extract and by dialysate but not by the ash of dialysate.

8. Cystine inhibited undialysed extract but not its undialysed equivalent. In dialysed extract it suppressed the activating effect of added amino-acids.

9. The following reactions were activated by amino-acids: phosphorylation of creatine by adenylpyrophosphate and by adenosinediphosphate. Phosphorylation of adenylic acid by creatine phosphate. Phosphorylation of arginine by adenylpyrophosphate in invertebrate muscle extract. Formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid. No effect could be shown on the formation of triosephosphate from hexosediphosphate.

10. Amino-acids have an effect on the solubility of sparingly soluble salts such as magnesium phosphate, magnesium adenylpyrophosphate, magnesium carbonate, calcium phosphate, magnesium and calcium phytates. This effect is particularly well marked at an alkaline reaction. It is brought about by the same compounds which activate phosphate transfer and all the compounds shown to have no effect there have no effect on the solubility of sparingly soluble salts.

11. Though ferric phosphate, ferric adenylpyrophosphate and ferric phytate formed soluble thiol complexes with cysteine, no effect could be observed with glycine.

12. It is pointed out that the effect of amino-acids on sparingly soluble salts may have a bearing on a number of physiological processes and should be kept in mind when the availability of a mineral *in vivo* is thought to be measured by its solubility in aqueous or acid extracts.

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78. The Effect of Protein Intake on the Absorption of Calcium and Magnesium

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Lehmann & Pollak [1942*a*] observed that the phosphates and carbonates of Ca and Mg were much more soluble in solutions of α-amino-acids than they were in pure water, and it has since been shown that the solubility of commercial 'phytin' can be increased in the same way. In consequence of their observations, Lehmann & Pollak suggested that amino-acids might facilitate the absorption of Ca. To prove or disprove this hypothesis, it was decided to study in human subjects the effect of varying protein consumption upon absorption and excretion of Ca and Mg.

Subjects and experimental arrangements

There have been five subjects, four men and one woman, and each has carried out two experiments, one at a high level and one at a low level of protein intake. A low protein and a moderately low Ca diet containing fixed rations of milk, 92% wheatmeal bread, sugar and table fat, was given in both experiments. The rest of the basal diet was made up essentially of potatoes and other vegetables, flavoured and cooked in various ways. The fare varied from day to day during the first experiment, but the menus were repeated exactly during the second. Each person weighed out and ate as much of the vegetable dishes as he or she wished during the first experiment, and adhered to the same amounts during the second.

These basal diets, which contained 45–70 g. of protein per day, were supplemented with 100–130 g. of protein (see Table 1) or with an equicalorific ration of sugar or fat. Four different sources of amino-acids were used to widen the scope of the investigation. All the protein supplements contained minerals, and, as far as possible, the intakes of Ca, Mg and P were made equal in the two experiments by administering a salt mixture with the sugar or fat supplement, or, alternatively, by reducing the milk ration when the protein intake was high. One of the protein supplements did not arrive in time to be analysed before the low protein experiment was well under way, and unfortunately in this case the Ca intakes were not equalized. The initials of the subjects, their ages, sex and weights, the duration of their experiments, the nature of their supplements and the

Table 1. *Particulars of subjects and experiments*

Initials, age and sex	Weight kg.	Duration of each exp. days	Nature and daily amount of protein supplement	Nature of non-protein supplement	Order of experiments
H.L. 31. M.	75	6	100 g. peptone ('Difco' proteose)	Sugar	High protein (H.P.) last
J.B. 25. M.	72	14	100 g. gelatin (Coignet's 'Extra')	Sugar	H.P. last
J.H. 32. M.	70	14	100 g. peptone (Turner's Commercial)	Margarine	H.P. first
P.C. 26. F.	67	14	130 g. gluten	Sugar	H.P. first
N.J. 27. M.	67·5	14	100 g. egg-white ('Jolo' brand)	Margarine	H.P. last

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order in which they were given may be found in Table 1. H.L.'s experiments were preliminary ones; no mineral supplements were given during the low protein period; he did not collect his faeces, and used his urinary excretion of Ca and Mg as a measure of his absorption of these elements [McCance & Widdowson, 1942*c*]. Lest the increased output of urea during the high protein experiment might increase the renal excretion of Ca or Mg [Aub, Tibbetts & McLean, 1937], H.L. took 30 g. urea by mouth on each day of his low protein experiment. The peptone supplements were dissolved in water and taken with food. Gelatin was made into very stiff jellies and eaten at meal times. Some of these jellies were flavoured with fruit juice. The dry gluten was incorporated with food such as mashed potatoes. At first the egg-white powder was dusted over other foods and eaten in this way, but the raw product seemed to provoke some discomfort and diarrhoea so, during the second week of the high protein experiment, it was dissolved in warm water and cooked. The experimental organization and all the technical details closely resembled those described by McCance & Widdowson [1942*a*] and need not be given again. H.L. passed straight from his low protein to his high protein diet but all the other experiments were preceded by a fore-period of 3 days and followed by the usual after-days.

Results

Table 2 shows the intakes, absorptions and urinary excretions of Ca at the two levels of protein consumption. The term 'absorption' is used throughout this paper to mean the amount of Ca in the food minus the amount in the faeces. It is recognized that rather more than this quantity must find its way out of the intestine because the digestive

Table 2. *The effect of varying the protein intake on the absorption and urinary excretion of calcium*

All the results are expressed as mg. Ca/day.

	Low protein experiment			High protein experiment		
Subject	Intake	Absorption	Urine	Intake	Absorption	Urine
H.L.	729	—	193	660	—	320
J.H.	700	30	83	663	83	117
P.C.	598	17	94	627	106	136
N.J.	603	49	40	585	92	67
Average for J.H., P.C. and N.J.	634	32	72	625	94	107

juices secreted into the gut must contain a certain amount of Ca, all of which will not be reabsorbed. This quantity, however, will be relatively constant on a fixed diet, and it does not detract from the practical value of using the term 'absorption' in the above sense. It will be observed that the amount of Ca ingested by the subjects was of the same order in each experiment, but tended to be lower when the protein intake was high. Yet the absorptions and urinary excretions were all raised by giving the protein supplements. J.B.'s results are not given in Table 2, for although he absorbed more Ca, as the others did, in his high protein experiment, the gelatin which he consumed was contaminated with Ca, and his intakes of Ca were therefore far from equal. It is to be noted that three of the four subjects in Table 2 absorbed very little of their dietary Ca when their protein intake was low. This was partly due to their own individual idiosyncrasies, for J.B. absorbed 96 mg./day out of an intake of 640 mg./day in his low protein experiment and H.L. probably absorbed twice this amount. Further, these experiments were carried out in March, when Ca absorption tends to be at its worst in certain individuals [McCance & Widdowson, 1943], and this may be one reason for the poor absorptions.

Table 3 shows the intakes, absorptions and urinary excretions of Mg at the two levels of protein consumption. The Mg intakes were approximately the same in the high and low protein experiments, and the averaged results were almost equal. The absorptions

Table 3. *The effect of varying the protein intake on the absorption and urinary excretion of magnesium*

All the results are expressed as mg. Mg/day.

	Low protein experiment			High protein experiment		
Subject	Intake	Absorption	Urine	Intake	Absorption	Urine
H.L.	547	—	179	522	—	223
J.H.	565	179	186	500	202	183
J.B.	561	206	170	605	284	239
P.C.	438	113	97	392	126	106
N.J.	505	163	159	580	227	192
Average for J.H., J.B., P.C. and N.J.	517	165	153	519	210	180

always rose when the protein intake was increased, and the urinary excretion of four of the five subjects rose also. If expressed as a percentage of intake instead of in absolute units, the urinary excretions rose in all five. The absorptions were not doubled or trebled, as those of Ca, but this was not surprising. Mg salts are generally more soluble than the corresponding Ca salts, and Mg ions tend to be much more freely absorbed from the intestine than Ca ions. Thus, on the low protein diet these subjects absorbed an average of 5% of their dietary Ca, but 32% of their dietary Mg. On the high protein diets the absorptions were 15 and 41% for Ca and Mg respectively. Each therefore was increased by about 10% of the intake. The average absorptions of Ca rose from 1·2 to 3·5 m.eq./day and those of Mg from 13·8 to 17·5, so that increasing the protein intake actually promoted the absorption of more Mg than of Ca ions.

Discussion

It is remarkable that the relationship between protein intake and Ca absorption has not been appreciated before. Lactose and fats have been reported to influence absorption, but Aub *et al.* were so dissatisfied with the state of knowledge existing in 1937 that they set out deliberately to search for the missing factor or factors in Ca absorption. They tried the effect of urea, with negative results, and the reason for this is now clear [Lehmann & Pollak, 1942*b*]. They did not, however, try the effect of protein. As a matter of fact, at least three workers or groups of workers have had results before them which demonstrated this influence of amino-acids on calcium absorption, but none of them quite realized the significance of what they had got. Mellanby [1921] observed, in the course of his studies of canine rickets, that lean meat 'had a definite anti-rachitic effect'. He recognized that its action in this respect was of a secondary nature and not comparable with that of the anti-rachitic vitamin. He did not determine the mechanism of this adjuvant anti-rachitic action of meat, but it is reasonable now to suggest that it was due to the protein it contained. The same explanation probably covers another observation of Mellanby [1925], namely, that separated milk had some anti-rachitic effect over and above that due to the Ca it contained. It must be admitted, however, that this was a subject of controversy before 1925, and has been ever since [Henry & Kon, 1939; Kempster, Breiter, Mills, McKay, Bernds & Outhouse, 1940]. Adolph & Chen [1932] compared milk and soya bean as sources of Ca to man at two levels of protein intake. Their metabolic periods were only 4 days in length and they had only three subjects, but their figures show that Ca was absorbed more freely when the protein consumption was high, and they noted that 'increasing the protein intake facilitated the attainment of Ca equilibrium'. They do not, however, seem to have pursued the matter further, and in one respect their results are a little puzzling, for they did not find that an increased absorption was always associated with an increased urinary excretion [McCance & Widdowson, 1942*c*]. This may just be an expression of the fact that their experiments were

rather slight and their periods too short for satisfactory metabolic work. Kunerth & Pittman [1939] and Pittman & Kunerth [1939] found that raising the N intake of three young women from an average of 4·01 to one of 10·92 g./day improved their Ca absorptions from 42 to 102 mg./day, and also increased the amounts excreted in the urine. The intakes of Ca averaged 458 and 436 mg./day in the two experiments so that the absorptions might have shown even more increase if the intakes had been exactly the same. Their metabolic studies were long ones and they commented on the fact that the 'high protein diet improved appreciably the utilization of ...Ca...by human subjects compared with the low protein diets of the earlier investigation'.

There have also been experiments on rats in which this effect of protein may have contributed to the results [Conner & Sherman, 1936; Conner, Kao & Sherman, 1939; Kao, Conner & Sherman, 1941], and others on pigs in which varying the protein intake was found to have no effect on mineral metabolism [Woodman, Evans & Turpitt, 1937]. These last experiments, however, were not arranged in the best possible manner for proving the point now being discussed, for the pigs were growing rapidly, Ca was probably not a factor limiting their growth, and the same animal does not seem to have been used for metabolic studies at both levels of protein intake. Hence absorptions at the two levels cannot really be compared.

Now that a relationship has been established between protein intake and Ca and Mg absorptions, the question at once arises whether P absorption is also improved, and whether Fe forms soluble and absorbable co-ordination compounds with amino-acids. Unfortunately the present studies do not provide a definite answer to either question. Of the three subjects whose Ca and P intakes were successfully equalized, the P absorptions of two (P.C. and J.H.) were improved by raising the protein intake, while that of the third (N.J.) was made slightly worse. J.B. absorbed the same amount of P in both his experiments. This suggests that protein was facilitating his P absorption, for, had it not been doing so, his absorption of P would have fallen off owing to his increased consumption of Ca from his gelatin. It seems possible, therefore, that protein does facilitate the absorption of P, but the effect is a small one.

The Fe intakes were not satisfactorily equalized in two of the subjects. The others showed no increased absorption when the protein intakes were raised, but it would be unwise to generalize from such limited material.

The recognition of the relationship between protein intake and Ca absorption clears up a number of other observations which have been made from time to time. It helps to explain why the Eskimos, who are largely carnivorous and whose Ca intakes cannot be large, tend to be well grown and have good teeth, and it provides an additional reason for recommending high protein diets in pregnancy and lactation. It makes the position of meat in the diet rather an interesting one, for this substance, admittedly a poor source of Ca, may so promote its absorption by providing a plentiful supply of amino-acids that it becomes the equivalent of a food rich in Ca [Mellanby, 1925]. Some of this effect may be neutralized by the phosphates which are present in meat and which themselves tend to inhibit the absorption of Ca [McCance & Widdowson, 1942*b*]. In H.L.'s experiments however, the large amount of phosphates in the peptone did not prevent a great increase in the absorption of Ca.

It is well known that individuals vary greatly in their ability to absorb Ca. These variations are not likely to originate as a rule in differences in protein consumption. J.B. for example, absorbed more Ca from an intake of 640 mg./day and a protein intake of 0·97 g./kg. of body weight/day than either J.H. or N.J. on similar intakes of Ca and protein intakes of the order of 2·4 g./kg./day. There is little doubt that H.L. absorbed more still, but the actual figures for his absorptions are not available.

Given that the level of protein intake is a factor in Ca absorption, the problem of assessing its quantitative significance is obviously a practical and important one. Clearly,

on the one hand, it provides a method of improving an adult's absorption of Ca without increasing his Ca intake and, since vitamin D in physiological doses does not seem to have this effect, juggling with the phytates and phosphates [McCance & Widdowson, 1942*a*, *b*] or raising the protein intakes would seem to be the only certain methods of doing so. If required, moreover, the protein intake can be varied with the Ca intake so that they will have a synergic action in promoting or impeding Ca absorption. There is a further point. If Tables 2 and 4 are considered, it can be shown that increasing the protein intake of J.H., P.C. and N.J. from an average of 55 to one of 165 g./day increased the Ca absorption from 32 to 94 mg./day. One might very well argue from this that these subjects would have absorbed no Ca at all in their low protein experiment had it not been for the protein in their basal diets. These considerations show that the protein in a diet may be an absolutely vital factor in Ca absorption. This is probably not the case in Mg absorption, for reasoning similar to the above suggests that on a protein-free diet the Mg absorptions would have been of the order of 140 mg./person/day. On the other hand, the control diets in these experiments were relatively low in protein and the supplements were large. The actual figures are given in Table 4. Normal diets in adult life

Table 4. *Protein intakes during the low and high protein experiments*

	Low protein diet		High protein diet	
Subject	g./day	g./kg. body wt./day	g./day	g./kg. body wt./day
H.L.	54	0·72	154	2·1
J.H.	60	0·86	160	2·3
J.B.	70	0·97	170	2·4
P.C.	45	0·67	175	2·6
N.J.	61	0·90	161	2·4

contain about 1 g. of protein/kg. of body weight, and it would be impracticable to double them, at any rate for long periods, even for therapeutic purposes. Consequently, varying the protein intake is unlikely in practice to bring about changes in absorption as great as those given in Tables 2 and 3. These are large, but they could probably have been achieved more easily—and much more cheaply—by increasing the mineral intakes. Thus, McCance & Widdowson [1942*a*] found that the addition of 0·1 g. Ca to 100 g. brown bread raised the average Ca absorption of five subjects, who were eating 1–1·5 lb. bread a day, from 43 to 149 mg. a day, and changing to a white bread diet produced about the same effect. This increase of 3–4 times is of the same order as that produced by 100 g. protein (Table 2).

It would seem, therefore, that while a good protein intake benefits Ca and Mg absorptions, and while it has been suggested that very little Ca would be absorbed from a protein-free diet, yet a high protein intake can never replace a satisfactory quantity of Ca itself, and should never even be considered as a substitute.

Summary

1. Metabolic studies on five healthy adults have shown that increasing the protein intake raised the amount of Ca and of Mg absorbed from the gut and subsequently excreted in the urine.

2. It is suggested that very little Ca would be absorbed if the diet contained no protein or amino-acids.

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79. The Absorption and Excretion of Zinc

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Zinc is now recognized to be one of the physiological elements, since it is essential for the growth of rats, and forms part of the carbonic anhydrase system [Shohl, 1939; Keilin & Mann, 1940]. Many foodstuffs contain comparable amounts of Fe and Zn, and the two metals tend to vary together. Thus white flour, egg-white and fish are poor sources of both, whereas brown flour, egg-yolk and meat are the reverse. There are, however, exceptions to this generalization. Milk contains three to five times as much Zn as Fe and the inside leaves of cabbage more Zn than the outer leaves [Sylvester & Hughes, 1936], whereas the reverse is true for Fe, and incidentally for Ca. Yeast, oysters and one or two exotic foods are very rich sources of Zn, but not necessarily of Fe.

The metabolism of Zn has not been fully worked out, although the subject has attracted attention from time to time, mainly owing to the extensive use of Zn in industry. Some excellent pioneering work was done by Röst & Weitzel [1918–19], Bachelor, Fehnel, Thomson & Drinker [1926], Drinker, Fehnel & Marsh [1927], and an informative review of the subject was published by Lutz [1926]. It had been established by that date that the normal human intake of Zn was of the order of 10–15 mg./day and that most of this was excreted in the faeces. Drinker *et al.* [1927] studied the Zn metabolism of two subjects for a week, in the middle of which a meal very rich in Zn was consumed. The urinary excretions did not rise, but within the next 24 hr. much additional Zn was passed in the faeces. Within the past 10 years the treatment of diabetics with Zn protamine insulin has been introduced and gained considerable popularity. The quantities of Zn administered are small, but they are given by subcutaneous injection and would amount to about 0·1 mg. of Zn per day in a patient who was receiving 50 units of Zn protamine insulin. The excretion of Zn after injection has been very little studied, although this seems the only way of finding out whether Zn is, or is not, freely excreted into the alimentary canal.

In 1938, metabolism experiments on six normal people were undertaken to study the fate of injected Ca, Mg, Fe and Zn. The way in which these experiments were done, and the results for Ca, Mg and Fe, have already been published [McCance & Widdowson, 1938; 1939]. In 1940–41 metabolism experiments were carried out to investigate the effects of brown and white bread on Ca absorption [McCance & Widdowson, 1942*a*]. These presented opportunities of making further experiments with Zn, and work on patients has also been possible from time to time. Some 45 Zn 'balances', each lasting for about a fortnight, have been carried out on twelve persons, and Zn has been injected intravenously into ten of them. Particulars about the subjects and the technical aspects of the experiments may be found in the papers to which reference has already been made. Zinc determinations were made on the ash extracts of foods, urines and faeces, the method used being that described by Keilin & Mann [1940].

Results

Zn balances at different levels of intake

Table 1 shows the Zn balances of three persons, one of them at two, and two of them at three, different levels of Zn intake. The low intakes were the result of specially planned diets. E.B.'s and E.W.'s moderate intakes were obtained when white flour constituted

Table 1. *Zn balances at different levels of intake*

Subject	Intake mg./day	Output, mg./day			Balance mg./day
		Urine	Faeces	Total	
E.W. ♀	5·6	0·4	5·2	5·6	±0·0
	9·9	0·4	9·6	10·0	−0·1
	19·6	0·3	18·2	18·5	+1·1
E.B. ♂	4·9	0·4	5·3	5·7	−0·8
	13·8	0·1	11·0	11·1	+2·7
	22·0	0·4	19·0	19·4	+2·6
R.H.E. ♂	6·1	0·3	6·0	6·3	−0·2
	9·1	0·3	8·5	8·8	+0·3

40–50 % of their total calories, the rest of the diet being mixed. Their high intakes were achieved by substituting wheatmeal of 92 % extraction for the white flour. R.H.E.'s higher intake was due to the ingestion of Zn salts. These results typify so well the many others which have been obtained that it is unnecessary to give the whole set. They show the subjects in approximate balance at all levels of intake. They show that the urinary excretions were exceedingly small, smaller in fact than those recorded by Röst & Weitzel [1918–19], Bachelor *et al.* [1926], Drinker *et al.* [1927] and Fairhall & Hoyt [1929]. The concentration of Zn in normal urine would seem to be of the same order as that in London tap water [Alport & Moon, 1939]. Scoular [1939] recorded still lower values, but since they were found in children the figures are not really comparable. Table 1 also shows that increasing the intake made no difference to the urinary excretion. This is a point of cardinal importance. It suggests that in normal life the kidney can take little or no part in regulating the amount of this metal in the body. Zn, therefore, resembles Fe in this respect and differs from Na, K, Ca and Mg, all of which are excreted by the kidney in amounts which vary with the requirements of the body and the amounts absorbed [McCance & Widdowson, 1942*b*].

The fate of injected Zn

In these experiments the intake by mouth and the output in the urine and stools have been measured for 4 or 5 weeks, and during the 3rd and 4th weeks 58–86 mg. Zn have been injected intravenously in twelve to fourteen divided doses. Most of the subjects have had only a single course of injections, but two had a second course after an interval. In the first experiment it was decided to use Zn protamine, and Messrs Boots very kindly prepared and presented a solution containing 2 mg. of Zn per ml. Unfortunately, the protein in this preparation produced unpleasant reactions on the second day, so inorganic Zn salts were substituted. These were dissolved in a convenient volume of 0·9 % NaCl and sterilized in the autoclave. The injections have been given at whatever hour of the day was most convenient and the time taken over them has never been less than 5 and has sometimes been as long as 40 min. In the earlier experiments Ca, Mg and Fe were given at the same time; in the later experiments the Zn was administered alone. These variations in the times and methods of administration have not appeared to influence the results in any way.

The results of nine experiments are given in Table 2. In one sense the subjects gave absolutely concordant results. The increases in urinary excretion were insignificant even when quite large amounts were injected. In R.M., M.M. and R.M.L. there was no increase, but Ca or Mg salts, injected at the same time, were quantitatively excreted [McCance & Widdowson, 1939]. Evidently, the kidney does not vary its excretion of Zn in accordance with the amount in the plasma or the amount absorbed. The traces constantly found in the urine may represent the end product of some quite different, possibly metabolic, function of the kidney. If so, the urinary excretion of Zn would be analogous to the intestinal excretion of Ca and Mg. Owing to the presence of

Table 2. *The fate of Zn given intravenously*

Subject	Intake, mg./day		Output, mg./day			Balance (excluding injected Zn) mg./day
	Mouth	Injection	Urine	Faeces	Total	
B.A. ♀	11·6	0·0	0·3	10·0	10·3	+1·3
	10·9	5·0	0·8	12·4	13·2	−2·3
E.B. ♂	5·7	0·0	0·5	6·7	7·2	−1·5
	5·7	6·0	0·9	9·0	9·9	−4·2
R.M. ♂	6·7	0·0	0·6	5·9	6·5	+0·2
	6·3	4·0	0·6	8·7	9·3	−3·0
K.B. ♀	10·7	0·0	0·5	10·3	10·8	−0·1
	12·4	6·0	0·8	11·6	12·4	∓0·0
R.M.L. ♂	5·8	0·0	0·4	5·5	5·9	−0·1
	6·7	4·0	0·4	6·3	6·7	∓0·0
R.H.E. ♂	6·1	0·0	0·3	6·0	6·3	−0·2
	7·1	4·0	0·4	7·8	8·2	−1·1
M.M. ♀	6·1	0·0	0·3	5·1	5·4	+0·7
	6·2	4·0	0·3	8·3	8·6	−2·4
E.W. ♀	7·8	0·0	0·4	7·6	8·0	∓0·2
	8·8	4·0	0·5	10·2	10·7	−1·9
Patient Ar. ♀	10·3	0·0	<0·1	9·2	9·3	+1·0
	11·8	6·2	0·3	17·1	17·4	−5·6

these two metals in the digestive juices, some excretion of both into the gut is inseparable from the alimentary functions of this organ. The amounts so excreted, however, are not ordinarily related to anything else, and certainly not to small changes in plasma concentration.

During the control periods four of the subjects were in slightly positive, and five in slightly negative, Zn balance. The averaged results for the group show an almost exact balance. Seven of the subjects responded to the injection of Zn by excreting more of the metal than they were obtaining in their food. Two did not, and the additional amounts put out by the others varied from one subject to another and were never as great as the quantities which had been injected. For this reason the results have not been averaged, and it was on account of this lack of uniformity in the response of the subjects that the experiments carried out in 1938 were not published at that time. The subjects, however, have not responded more uniformly in the later experiments, and it is now felt that these irregularities are physiological, not technical, in origin. It would seem, therefore, that the human body possesses a means of getting rid of an excess of Zn from its tissues, but that the sensitivity of the regulating mechanism to a given dose varies with the individual and possibly with the degree of saturation of his storage organs. It is interesting to find the intestinal route the one selected by Nature for the excretion of a physiological metal. Owing to its absorptive functions the intestine can never achieve such a fine adjustment as the kidney, and can only act effectively for a non-toxic ion likely to present itself in very small amounts and forming relatively insoluble salts. Mn is such a metal, and it seems to be excreted very much like Zn [Kent & McCance, 1941]. Fe has the requisite properties, but does not appear to be excreted at all. It is interesting to picture the state of affairs which would result if the large intestine was the only part of the body which could deal with the excretion of Na salts.

From the small number of papers which have been published on the subject, it is probable that the response of animals to Zn injections will be found to be similar to that of human beings. Salant, Rieger & Treuthardt [1918] injected Zn into rabbits and cats in quantities relatively greater than those used in the present experiments, and subsequently found very little in the urines, but quantities up to half the amounts injected in the intestinal tracts. It is not clear from their paper, however, whether due allowance was made for the Zn in the animals' food, and particularly in that of the rabbits.

The excretion of Zn in patients with albuminuria

Since Zn salts will readily precipitate plasma proteins, it must be supposed that most of the Zn in the plasma is so combined. This would explain why the urine contains so little Zn, and why injecting Zn does not provoke any excretion of the metal in the urine. The same explanation probably underlies the fact that normal urines are practically Fe-free. If this is the case, patients with albuminuria might be expected to excrete more Zn in their urines than normal persons. Fairhall & Hoyt [1929] made a survey of this subject and came to the conclusion that the amount of Zn in the urine bore no relationship to the degree of albuminuria, but they did find high concentrations of Zn in the urine of nephritics. They noted, however, that the daily excretions of such patients were not greater than those of normal subjects and they commented on the possibility that the daily outputs of metal might have been abnormally high, had the volumes of urine passed by the nephritics not been so low. It was decided to re-examine the matter, and measurements were made of the daily excretion of Zn by a number of patients with moderate and heavy albuminuria. The greatest care was taken to ensure that the patient, bed, bedding and collecting vessels were free of all ZnO or dusting powder. The figures were far outside the range of those of the present series obtained from normal persons. The actual figures for six patients ranged from 1·0 to 3·8 and averaged 2·1 mg./day, whereas the normal ones varied from 0·11 to 0·50 and averaged 0·29 mg./day. Thus the urinary excretions were about seven times the normal size in patients with albuminuria. It was confirmed, however, that the amount of Zn excreted was not directly correlated with the degree of albuminuria. Determinations of the plasma Zn might have helped to establish some correlation, but unfortunately none were made. The urinary excretion of vitamin A may be recalled in this connexion [Lawrie, Moore & Rajagopal, 1941]. The vitamin is excreted only by patients with protein or proteose in the urine, but there is no quantitative correlation between the two excretions.

In normal persons the urinary excretion of Zn (as of Fe) may almost be neglected in balancing up intakes and outputs, but this is not the case in some patients with albuminuria. A study of such persons offered a method of finding out something about the ease with which Zn was absorbed and also about the intestinal exchanges. One would expect that, if Zn were freely absorbed and the surplus excreted back into the gut, patients who were losing much Zn in their urines would excrete less Zn in their faeces than they took in their food, and not as normal subjects almost the same amount. Balance experiments, therefore, were carried out on two patients and the results are shown in Table 3. It will be seen that neither subject appeared to be absorbing any Zn,

Table 3. *Zn balances of patients with heavy albuminuria*

Subject	Intake mg./day	Output, mg./day			Balance mg./day
		Urine	Faeces	Total	
M.E.	13·9	3·0	15·3	18·3	−4·4
H.E.	13·7	2·3	13·8	16·1	−2·4

yet M.E. was losing 3 mg. and H.E. 2·3 mg./day in the urine. One must conclude from these limited experiments that Zn is not freely absorbed and that patients with albuminuria may not absorb the metal rapidly enough from ordinary diets to make good their urinary loss. Indeed, if they were to live long enough, they might even become short of Zn. Symptoms of Zn deficiency in human beings have, however, never been described. If it is true that adults absorb very little Zn, then very little must normally be excreted into the gut. This function, therefore, is only exerted strongly in the abnormal circumstances occasioned (in these experiments) by an injection of Zn.

SUMMARY

1. 45 Zn balances have been carried out on normal persons and on patients.

2. Normal persons excreted about 0·3 mg./day in the urine. This amount did not vary with the intake by mouth, nor was it appreciably raised by intravenous injections.

3. Patients with albuminuria excreted about seven times the normal amount of Zn in their urine.

4. Within the limits of experimental error, normal adults excreted almost the same amount of Zn in their stools as they ingested in their food. Some adults excreted additional Zn in their faeces when amounts up to 6 mg./day were injected intravenously for a fortnight; others did not.

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80. Palm Oil Carotenoids

2. The Isolation of Lipoid Pigments from a West African Plantation Oil and some Remarks on the Isomerization of Carotenoids

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As already mentioned [Hunter & Scott, 1941], it is to be anticipated that the carotenoids present in palm oils will vary to some extent with the district of origin and the previous history of the oils concerned. In general, African palm oils may be classified into two main groups: (i) oils of 'native' origin, and (ii) plantation oils of low free fatty acid content which are subject to greater care in production. Those of the second class might therefore be expected to show much smaller variations than the former [Strain, 1941], and to obtain information with regard to the carotenoids of an oil of the second type, a study has been made of a palm oil from a West African plantation.

A number of samples of oil from the N'dian Oil Palm Estate, which were specially imported in small amounts for this purpose, were examined as described in the first paper. Chromatographic adsorption of the petroleum-soluble unsaponifiable matter of the first of these samples of oil on a Tswett column of alumina gave a series of coloured zones, the lower two of which furnished crystalline and reasonably pure specimens of α- and β-carotene. The upper zones yielded carotenoids having the spectroscopic characteristics of lycopene and *neo*lycopene, which exhibited the reversible isomerization described by Zechmeister & Tuzson [1938].

The ether-soluble unsaponifiable matter on being dissolved in light petroleum and partitioned with 90% methanol gave a hypophasic fraction which on chromatographic adsorption on alumina furnished a small amount of lutein. The absorption maxima of the gum obtained from the coloured zone above this corresponded with those of *neo*lutein [Zechmeister & Tuzson, 1938].

The sterol fraction of the ether-soluble unsaponifiable matter also yielded a small amount of ergosterol, which was recognized from its ultra-violet absorption spectrum. This observation seems of particular interest in view of the presence of ergosterol in the unsaponifiable matter of grass [Gillam, Heilbron, Morton, Bishop & Drummond, 1933], soya bean oil [Izume, Yoschimaru & Hikada, 1933], cotton seed oil [Windaus & Bock, 1937], and corn germ oil [Lobert, 1938].

From the petroleum-soluble unsaponifiable matter of a second palm oil of slightly lower free fatty acid content but similar spectroscopic absorption, in addition to the isolation of a small specimen of lycopene of high extinction coefficient, evidence was obtained of the presence of γ-carotene.

With regard to the view of Zechmeister & Tuzson that the reversible isomerization of carotenoids is due to 'mobile' *cis-trans* isomerism, attention has already been drawn [Jones, 1940] to the fact that in the cases of the polyene acids, bixin and crocetin and their derivatives which are known to exist in *cis* and *trans* modifications [Kuhn & Winterstein, 1932], the more labile *cis* isomers show absorption spectra maxima displaced towards the region of shorter wave-length to an extent comparable with those observed in *neo*-carotenoids. Mulliken [1939] considers, moreover, that the conjugation of the carbonyl group and doubly linked carbon groups exerts a special stabilizing influence in such cases. Quantum mechanical considerations indicate that conjugated systems tend

to remain coplanar [Pauling, 1940], and Mulliken [1939] concludes that the ordinary and more stable form of carotenoids is the most elongated structure with *trans-trans* linkages [cf. Zechmeister, Le Rosen, Went & Pauling, 1941]. The labile *neo*-isomers presumably contain at least one *cis-trans* linkage.

The analogy with azobenzene [Hartley, 1938], although differing in that the *cis* isomer is not coplanar [Robertson, 1939] owing to steric hindrance, seems highly suggestive. The ordinary form of azobenzene whose *trans* configuration follows from its zero dipole moment [Bergmann, Engel & Sandor, 1930] and the X-ray analysis of the crystal structure [Robertson, Prasad & Woodward, 1936; de Lange, Robertson & Woodward, 1939], may be converted into a labile *cis* form which can be separated chromatographically [Cook, 1938]. Dilute solutions of the same concentration of either isomer furnish an equilibrium mixture containing 15–40 % of the *cis* form, depending on the solvent. It is noteworthy that all the carotenoid isomerizations so far recorded have taken place in non-polar solvents, in which Hartley [1938] observed rapid isomerization of azobenzene. We have now shown that β-carotene isomerizes more rapidly in toluene (non-polar) than in nitromethane (polar) solution, thus paralleling the latter observations.

The original theory that the isomerization of carotenoids is due to three-carbon prototropy with movement of a terminal double bond out of the conjugated system [Gillam & El Ridi, 1936] is ruled out, not only by the arguments adduced by Zechmeister *et al.* [1941], but by the fact that α-carotene gives rise to an isomer, *neo*-α-carotene, with retention of optical activity [Gillam, El Ridi & Kon, 1937]. The tautomeric system is of the true three-carbon type (as distinct from the pentad keto-enol system) such as is present in the diphenylpropenes which were first thought to be static [Ingold & Piggott, 1922], and only undergo interconversion under most stringent conditions [Ingold & Shoppee, 1929].

Experimental

The first oil examined was a small delivery which had a free fatty acid content of 3·5 % and which showed an extinction coefficient of 2·65 at 458 mμ in chloroform. A number of experiments were made with this oil, of which the following are typical.

(i) The palm oil (200 g.) was heated under reflux with a mixture of aqueous KOH (56 %, 200 g.) and industrial alcohol (600 ml.) for 1½ hr. and the resulting soap solution was diluted with a further 600 ml. of alcohol, made up to 4 l. with water, and thrice extracted with light petroleum. The united extracts were thrice washed with 90 % methanol (total volume 500 ml.), thereafter with water until free from alkali, dried with anhydrous Na_2SO_4, and freed from solvent under reduced pressure in an atmosphere of nitrogen.

The petroleum-soluble unsaponifiable matter was resaponified by heating with a mixture of alcoholic NaOH (3 g. of NaOH in 250 ml. of 90 % alcohol) and benzene (50 ml.) for 2 hr., and the benzene was removed by distillation. The mixture was diluted with 50 % alcohol (500 ml.) and thrice extracted with light petroleum, the united extracts washed and evaporated, and a solution of the residue in light petroleum was adsorbed on a Tswett column of 'Birlec' alumina. The chromatogram furnished a narrow dark red zone (1), below the usual 'oxidized carotenoid zones', whose appearance was suggestive of lycopene, followed by a second narrow orange brown zone (2) which separated from the broad carotene zone (3). Elution of zone (1) gave 0·004 g. of a carotenoid which showed absorption maxima at 539, 501 and 469 mμ in carbon disulphide and evidently consisted of impure lycopene. Zone (2) furnished 0·009 g. of material showing absorption maxima at 526, 491·5 and 462 mμ in carbon disulphide and a band at 590 mμ in the antimony trichloride reaction. The carotene zone (3) on being rechromatographed on Merck's alumina furnished crude β-carotene whose solution in ethyl acetate furnished crystals, M.P. 158°, on keeping in a refrigerator. On recrystallization these gave glistening red-brown crystals, M.P. 181–182°, which showed absorption maxima at 516 and 485 mμ

in carbon disulphide and at 495 and 463 mμ in chloroform. The $E^{1\%}_{1\,cm.}$ value at 463 mμ in chloroform was 2080.

(ii) The unsaponifiable matter from 3 kg. of the oil was worked up in batches as follows. The oil (1000 g.) was heated with NaOH (33%, 840 g.) and industrial alcohol (3·3 l.), under reflux, for an hour and the solution was diluted with alcohol (3 l.) and water (12·6 l.). 4 l. of a mixture of light petroleum and methylated ether (1 : 1) were added and the mixture was introduced into an extraction column packed with 1½ in. Lessing porcelain rings and the extraction was continued for 4 hr. The united extracts were washed and the residue obtained by removal of solvent was resaponified by heating with a mixture of alcoholic NaOH (10 g. of NaOH in 400 ml. of 90% alcohol) and benzene (80 ml.) for 5 hr. The residue obtained by removal of benzene was extracted five times with light petroleum in the presence of 50% aqueous alcohol (1000 ml.), and the united extracts were washed with 90% alcohol and water and dried with anhydrous Na_2SO_4. The unsaponifiable matter obtained by removal of solvent weighed 7·49 g.

On treatment with a small amount of acetone, the unsaponifiable matter deposited a solid 'A' (0·92 g.). The filtrate from this on evaporation and adsorption of a solution of the residue in light petroleum on a Tswett column of 'Birlec' alumina gave a series of narrow upper zones (orange-brown, red-brown, orange-brown, orange-red, orange-yellow, orange-red, and yellow, in order of decreasing adsorption), all of which gave substances showing indefinite absorption spectra in the visible region, followed by a broad orange-red zone containing lycopene. Extraction of this with acetone furnished a solid 'B' (0·2 g.) which showed maxima at 546, 507, 474 and 452 mμ in carbon disulphide and at 520, 485, 455 and 427 mμ in chloroform, in agreement with recorded data for lycopene.

The orange-yellow *neo*lycopene zone below this furnished 0·167 g. of a substance whose solution in benzene-methanol gave, however, a small amount of material showing the spectroscopic characteristics of lycopene (maxima at 545, 505 and 475 mμ in carbon disulphide and at 516, 483 and 457 mμ in chloroform). The mother liquors from this, on evaporation and further chromatography on 'Birlec' alumina also gave material having the spectroscopic characteristics of lycopene (maxima at 543, 505 and 475 mμ in carbon disulphide). The mother liquors from the original lycopene extract on evaporation and chromatography on 'Birlec' alumina furnished two main zones, the upper one of which had an orange-pink colour and furnished lycopene (maxima at 545, 507 and 476 mμ in carbon disulphide). The lower orange-coloured zone gave a substance showing maxima at 536, 499 and 472 mμ in carbon disulphide. Zechmeister & Tuzson [1938] record 536, 498 and 466 mμ for the absorption maxima of *neo*lycopene in carbon disulphide.

The broad carotene zone below *neo*lycopene consisted of a mixture of α- and β-isomers in which the latter predominated. The solid 'A' which originally separated on treatment of the unsaponifiable matter with acetone consisted mainly of a mixture of α- and β-carotenes. A solution of this in light petroleum on adsorption on a column of Merck's alumina gave a chromatogram consisting of a small narrow dark red upper zone, followed by a broad typical β-carotene zone and a smaller lighter coloured zone containing the α-isomer. On elution with acetone the β-carotene zone furnished crops of crystals melting at 182° and 179°.

(iii) The oil (1500 g.) was saponified in two batches of 750 g. as described for 'Sherbro' palm oil in our first paper. The heat of the reaction enabled a temperature of about 44° to be maintained without external heating for some time. The united petroleum extracts (*ca.* 1500 ml.) were washed, dried and evaporated under reduced pressure in an atmosphere of CO_2, the temperature being kept below 50°.

The extracted soap solution was diluted with water (3350 ml.) and extracted with ether. The ether-soluble unsaponifiable matter (3·53 g.) was dissolved in light petroleum (250 ml.) and the solution extracted 10 times with 90% methanol (total volume 500 ml.); the united extracts were diluted with water (1000 ml.) and thrice extracted with ether.

The residue obtained by removal of ether was dissolved in light petroleum and adsorbed on a column of 'Birlec' alumina, and the chromatogram was developed with petroleum containing 0·5 % of methanol. Four zones were obtained (orange, yellow, orange-brown, and red-brown in order of decreasing adsorption) which were separated, eluted and separately re-chromatographed. The carotenoid from the upper zone of the original chromatogram now gave a well-defined main zone on being developed with light petroleum containing 3 % of benzene. On elution this gave a small amount of solid which showed maxima at 507·5, 476 and 447 mμ in carbon disulphide and at 489, 458·5 and 431 mμ in chloroform. The antimony trichloride reaction gave a maximum at 620 mμ which shifted to 623 mμ, and a solution of the carotenoid also gave a blue colour in conc. H_2SO_4 which turned green on pouring into water [Zechmeister, 1934]. Gilman [1938] gives the absorption maxima for lutein in carbon disulphide as 508, 475 and 445 mμ, and Gillam [1935] recorded the maxima of lutein (from nettles) in chloroform as 489, 456 and 428 mμ.

The second zone furnished a gum which showed absorption maxima at 502, 473 and 442 mμ in carbon disulphide and at 484, 454 and 431 mμ in chloroform. A solution of the gum in methyl alcohol furnished a trace of solid which gave absorption maxima at 508, 477 and 446 mμ in carbon disulphide and at 488, 457 and 430 mμ in chloroform. Zechmeister & Tuzson [1938] record maxima at 501 and 468 mμ for *neo*lutein A and at 502 and 470 mμ for *neo*lutein B in carbon disulphide. The third zone of the original chromatogram gave no substance of distinctive properties. The fourth zone (red-brown) on being re-chromatographed furnished a gummy solid which showed a vague maximum at 475 mμ in carbon disulphide, and vague maxima at 487 and 460 mμ in chloroform.

The petroleum-soluble unsaponifiable matter (4·33 g.) was resaponified at 40° for 4 hr. and its solution in light petroleum was thereafter adsorbed on a column of 'Birlec' alumina. The dark red zone above the main carotene zone on being re-chromatographed furnished a small amount of crystalline lycopene (absorption maxima at 547, 507 and 478 mμ in carbon disulphide and at 520, 485 and 455 mμ in chloroform). The main carotene zone on further chromatography on alumina ('Birlec' and thereafter British Drug Houses Ltd., using a 20 % solution of benzene in light petroleum for development) furnished α-carotene which separated from benzene-methanol in crystals melting at 184°. This had an $E^{1\%}_{1\,cm.}$ value of 2270 at 460 mμ in chloroform and a rotation of $[\alpha]_D = +600°$ in benzene.

The first part of the percolate obtained during chromatography of the main carotene zone furnished a small amount of waxy material which separated from acetone in colourless crystals, M.P. 54° which rose to 57–58° on recrystallization, and which was evidently hentriacontane [Hunter & Scott, 1941].

(iv) In an experiment similar to (i) in which 500 g. of the palm oil were saponified by heating with aqueous KOH (56 %, 500 g.) and industrial alcohol (1500 ml.) for an hour, the ether-soluble unsaponifiable matter was dissolved in light petroleum to give a solution which deposited an orange-coloured powdery material (M.P. *ca.* 150°) on keeping. The filtrate from this, on adsorption on alumina, gave a series of zones (yellow, brown, orange, red-brown, pale orange, etc. in order of decreasing adsorption), the second of which, on extraction with acetone, furnished crops of pale yellow crystals, M.P. 139° and M.P. 140°. The mother liquors on evaporation and crystallization of the residue from light petroleum gave a further crop of crystals, M.P. 137°. The combined crops on recrystallization from acetone furnished transparent plates, M.P. 139·5°. A solution of this substance in alcohol showed the ultra-violet absorption spectrum of ergosterol with maxima at 250·5, 260·5, 270, 281 and 293 mμ. Hogness, Sidwell & Zscheile [1937] record absorption maxima at 252, 262, 271, 282 and 293 mμ for ergosterol in ethyl alcohol. The $E^{1\%}_{1\,cm.}$ value at 281·5 mμ was, however, only 7·7, indicating the presence of only some 2·5 % of ergosterol.

The residue obtained by evaporation of the mother liquors from the acetone solution, on being dissolved in alcohol and treated with digitonin in the same solvent, gave a precipitate which was washed with alcohol and ether and thereafter heated, under reflux, with acetic anhydride (25 ml.) for an hour. The residue obtained by removal of acetic anhydride by distillation, on recrystallization from dilute alcohol ('norit') furnished crystals melting at 131°.

The second palm oil examined was a 5 cwt. drum delivery which had a free fatty acid content of 2·8 % and showed an extinction coefficient of 2·6 at 458 mμ in chloroform.

1500 g. of this oil were saponified in two batches as in (iii) and the petroleum-soluble unsaponifiable matter was adsorbed on 'Birlec' alumina, using a 25 % solution of benzene in light petroleum as solvent and for development. The chromatogram formed a series of zones: (1) brown, (2) dark red, (3) red-brown, (4) ochre, (5) typical carotene and (6) narrow pale yellow, in order of decreasing adsorption. The lowest zone and part of the carotene zone were washed out of the Tswett column. Evaporation of the former and crystallization of the residue from acetone gave a small amount of hentriacontane melting at 67–68°. The brown zone (1) yielded material showing indefinite absorption in carbon disulphide. Zone (2) on being re-chromatographed on 'Mayfair' alumina, using 50 % benzene in light petroleum for development of the new chromatogram gave a main zone containing lycopene. A small specimen of this which crystallized from benzene-methanol, although it had a low melting point (160°), showed well-defined absorption maxima at 518, 485 and 454 mμ in chloroform with the corresponding $E^{1\%}_{1\,cm.}$ values of 2070, 2380 and 1610. In *cyclo*hexane, it showed maxima at 509, 476 and 448 mμ with $E^{1\%}_{1\,cm.}$ values of 2100, 2420 and 1620 respectively. Gillam [1935] recorded a figure of only 2000 for the $E^{1\%}_{1\,cm.}$ value of lycopene at its main absorption band in chloroform. The smaller zone below this furnished a carotenoid showing maxima at 535, 496 and 465 mμ in carbon disulphide, which was probably *neo*lycopene.

Zone (3) on being re-chromatographed on 'Mayfair' alumina gave a main orange-red zone which furnished a carotenoid showing absorption maxima at 529, 494 and 463 mμ in carbon disulphide which was probably γ-carotene [Hunter & Scott, 1941]. A dark red zone which formed below this gave a substance having almost identical spectroscopic characteristics (maxima at 529, 494 and 458 mμ).

Zone (4) on being re-chromatographed gave a yellow zone between more coloured zones which furnished a carotenoid showing maxima at 454 and 426 mμ in carbon disulphide.

The ether-soluble unsaponifiable matter was dissolved in light petroleum and the solution was extracted with 90 % methanol as in previous experiments. On adsorption on 'Birlec' alumina, using 50 % benzene in light petroleum, a chromatogram consisting of five zones was obtained (brown-orange, narrow brown, red, orange and yellow, in order of decreasing adsorption). Elution of the first three of these gave substances having very indefinite absorption spectra, but the orange zone below them gave a carotenoid showing absorption maxima at 503 and 472 mμ in carbon disulphide. This gave a purple-blue colour without selective absorption in the antimony trichloride reaction. The yellow zone below this yielded a carotenoid showing maxima at 502 and 473 mμ in carbon disulphide, which gave a transient band at 590 mμ in the antimony trichloride reaction.

Experiments on the isomerization of β-carotene

Crystalline β-carotene (*ca.* 5 mg.) was dissolved in carbon disulphide (*ca.* 0·8 ml.); 0·15 ml. of this solution was added to nitromethane (2 ml.) and the mixture was heated in a water bath with constant shaking for 3 min. The solution was then rapidly evaporated to dryness on a Hyvac pump fitted with a CO_2 trap, and the residue was dissolved in carbon disulphide and examined on the Hilger-Nutting spectrophotometer.

A similar experiment was made with 0·15 ml. of the β-carotene solution in carbon disulphide and 2 ml. of toluene.

The results were as follows:

	Absorption maxima mμ	
Original β-carotene solution	516,	485
After treatment with nitromethane	505–510,	483
After treatment with toluene	—	477–478

The solutions of carotene which had been treated with nitromethane and toluene were evaporated and the residues chromatographed on 'Mayfair' alumina, using a 20% solution of benzene in light petroleum as solvent. The carotene which had been treated with nitromethane yielded three zones (narrow orange, pink, and orange) which on elution gave substances with the following spectroscopic characteristics in carbon disulphide:

	Absorption maxima mμ
Zone 1	494
Zone 2	510, 483
Zone 3	510, 483

The carotene which had been treated with toluene gave two zones of similar appearance to zones 1 and 3, but only the second of these gave sufficient material for examination, and showed maxima at 505–510 and 481 mμ in carbon disulphide.

Summary

1. Examination of the unsaponifiable matter of a West African plantation oil of low free fatty acid content showed the presence of α-, β- and γ-carotenes, lycopene, *neo*lycopene, lutein, *neo*lutein, and the carotenoid intermediate between γ- and β-carotenes on the chromatogram which was previously observed in 'Sherbro' palm oil.

2. Evidence was also obtained of the presence of ergosterol.

3. Analogy is drawn between the reversible isomerization of carotenoids and the 'mobile' *cis-trans* isomerism of azobenzene.

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